11-30-2017

The Regulation Of Lactate Metabolism In Epithelial-Mesenchymal Transition Of Human Breast Cancer Cells

Denisse Tafur
University of Connecticut - Storrs, denisse0203@hotmail.com

The Regulation Of Lactate Metabolism In Epithelial-Mesenchymal Transition Of Human Breast Cancer Cells

Denisse Guadalupe Tafur-Cotrina, Ph.D.

University of Connecticut, 2017

Lactate is both a metabolite of glycolysis, and a component of several signaling pathways. Although recent studies indicate that lactate is a critical regulator of cancer development, very little is known about lactate metabolism in the context of metastatic cancer. Epithelial-to mesenchymal transition (EMT) promotes metastasis by inducing invasive properties in epithelial tumors. To determine whether EMT induces metabolic alterations, we have studied two epithelial breast cancer cell lines (MCF-7 and BT-474) and their respective EMT-induced mesenchymal progeny (MCF-7 M and BT-474 M) for changes in lactate metabolism. Metabolic analysis revealed that EMT induced an enhanced glycolytic profile in mesenchymal breast cancer cells along with the overexpression of glucose transporters (GLUT3, GLUT12), lactate dehydrogenases (LDHA, LDHB) and monocarboxylate transporter 4 (MCT4). In contrast, epithelial breast cancer cells preferentially use oxidative phosphorylation (OXPHOS) to produce ATP. These aerobic epithelial breast cancer cells expressed the monocarboxylate transporter 1 (MCT1) and maintained significantly higher intracellular levels of lactate and pyruvate. The strong expression of the lactate importer MCT1 in epithelial breast cancer cells may involve the ERα-inducible GATA3 transcription factor. GATA3 is central to normal breast development, and is expressed in ERα positive breast cancer. Using the UCSC Genome Browser, we detected a specific GATA3 binding

site in the DNAse-hypersensitive region within the promoter of MCT1 gene, and found that knockdown of GATA3 represses MCT1 expression. We also observed that the endogenous lactate receptor, G-protein coupled receptor 81 (GPR81), was highly expressed in epithelial breast cancer cell lines and hormone-positive breast cancer tumors but was suppressed in post-EMT mesenchymal cancer cells or triple negative breast cancer (TNBC) tumors. GPR81 regulated MCT1 expression and lactate uptake in epithelial breast cancer cells and its expression was crucial for cell proliferation and survival under nutrient limited conditions. This study provides an overview of specific metabolic changes induced by EMT in two independent and substantially different epithelial breast cancer cell lines. In addition, we propose GPR81 as a potential prognostic marker and therapeutic target in hormone positive epithelial breast cancer.

The Regulation Of Lactate Metabolism In Epithelial-Mesenchymal Transition Of Human Breast Cancer Cells

Denisse Guadalupe Tafur-Cotrina

B.S. Universidad Peruana Cayetano Heredia, 2010

A Dissertation

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

at the

University of Connecticut

2017

APPROVAL PAGE

Doctor of Philosophy Dissertation

The Regulation Of Lactate Metabolism In Epithelial-Mesenchymal Transition Of Human Breast Cancer Cells

Presented by

Denisse Guadalupe Tafur-Cotrina, B.S.

Major Advisor

Bruce A. White

Associate Advisor

Molly A. Brewer

Associate Advisor

John R. Harrison

Associate Advisor

Lisa M. Mehlmann

University of Connecticut

2017

DEDICATION

This thesis is dedicated to my beloved parents Silvia Cotrina Cuizano and Jaime Tafur Valqui for their love, endless support, guidance and encouragement to pursue my dreams. I will never finish to thank them for all the sacrifices they made for my sister and me to give us the best in every possible way.

To my sister and unconditional friend Carla Tafur for her love and encouragement when I needed the most.

To my soulmate Adam Lafontaine, for his love, friendship and constant support during the past five years of our doctoral journey.

and to my beloved Leela

ACKNOWLEDGMENTS

First of all, I would like to express my deepest and most sincere gratitude to my mentor Dr. Bruce White, for his continuous support throughout my Ph.D. I will always be thankful to him for his guidance, patience, enthusiasm and immense knowledge which made me a better scientist and helped me to reach my goals. I could not have imagined having a better mentor. I also want to thank my former labmate Dr. Yuva Kondaveeti for his support, encouragement and friendship.

I would like to thank the members of my thesis committee, Dr. Molly Brewer, Dr. John Harrison and Dr. Lisa Mehlman, for their encouragement, constructive comments and support. I would like to thank Dr. Laurinda Jaffe and Dr. Mark Terasaki for their generous financial support for this work.

I would like to thank my college professors Dr. Mirko Zimic and Dr. Patricia Sheen for giving me my first research project that allowed me to discover my career path. I want to express my gratitude to Dr. Abel Alcazar, Dr. Mateus Guerra and Dr. Michael Nathanson for their support, guidance and encouragement to pursue a Ph.D.

I would like to thank my friends Leia, Karina U., Karina V., William, Laura, Lane, Jack, Abhijit, Sofia and Maria Jesus for their love and support throughout these years.

Also, I would like to thank my extended family in Peru especially to my grandparents for their love, understanding and support to pursue my dreams even though that meant to be far away from them.

Finally, I would like to thank the Lafontaine family for their love, caring and making me feel at home. I am so blessed to be part of their family.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

EMT	epithelial–mesenchymal transition
MET	mesenchymal-epithelial transition
TNBC	triple negative breast cancer
OXPHOS	oxidative phosphorylation
ECAR	extracellular acidification rate
ROS	reactive oxygen species
GATA3	GATA binding protein 3
ZEB1	zinc finger E-box binding homeobox 1
ZEB2	zinc finger E-box binding homeobox 2
ER	estrogen receptor
PR	progesterone receptor
HER2	amplified ERBB2 oncogene
GPR81	G protein-coupled receptor 81
GPR109A	G protein-coupled receptor 109A
GPR109B	protein-coupled receptor 109B
MCT1	monocarboxylate transporter 1
MCT2	monocarboxylate transporter 2
MCT4	monocarboxylate transporter 4
LDHA	lactate dehydrogenase A
LDHB	lactate dehydrogenase B
GLUT1	glucose transporter 1
GLUT3	glucose transporter 3

GLUT12	glucose transporter 12
PCK2	phosphoenolpyruvate carboxykinase 2
FBP1	fructose-1,6-bisphosphatase 1
G6PD	glucose-6-phosphate dehydrogenase
PCNA	proliferating cell nuclear antigen
STK15	aurora kinase A

CHAPTER 1

GENERAL INTRODUCTION

I. THE BREAST

i. Anatomy

The female breast represents a gland that is specialized in the synthesis, secretion and delivery of milk to the newborn. This complex tissue is composed of lobules (milk producing glands), ducts (milk-carrying tubes), blood vessels, lymph nodes and stroma (adipose and connective tissue surrounding the ducts and lobes). The breast is organized into 15 to 20 lobes, each lobe has 20 to 40 lobules which consist of clusters of alveoli containing mammary secretory epithelial cells. Lobules are connected to very small ducts that merge into a unique duct for each lobe (2-4.5 cm long) that ends in its own opening at the nipple. The dark area of skin surrounding the nipple that contains sweat and sebaceous glands is called the areola. Although the breast is mature after puberty, the breast tissue remains inactive until pregnancy[1,2].

ii. Development

The breast undergoes many changes in structure and function during a woman's lifetime in response to hormonal stimuli[1]. From birth through childhood, the mammary gland remains small and immature[3]. Later during puberty, hormones produced by the ovaries and pituitary gland cause the breast to grow and mature. This maturation process is called branching morphogenesis and involves enlargement of the milk ducts by expansion of the epithelial ductal cells[4]. During pregnancy, prolactin and placental lactogen induce the alveolar epithelial cells to proliferate and differentiate into milk-secreting cells. During lactation, milk is secreted by epithelial cells and accumulated in the lumen of the alveoli. Oxytocin, stimulated by suckling infant, causes the contraction of the surrounding myoepithelial cells that moves the milk through the ductal tree and to the nipple. Upon weaning, the reduction of hormonal levels stops milk production and promotes apoptosis of epithelial cells, an event referred to as involution[1,4].

II. BREAST CANCER

i. Epidemiology

Breast cancer is the most commonly diagnosed cancer in women and it is one of the leading causes of cancer deaths in the world. According to the World Health Organization, breast cancer was responsible for 571,000 deaths in 2015. In the US, the National Cancer Institute estimates 255,180 new cases (15% of all new cancer cases) along with 41,070 deaths (6.8% of all cancer deaths) for breast cancer in 2017. The incidence of breast cancer and mortality increase dramatically with age. Breast cancer is most frequently diagnosed in middle-aged and older women among women aged 55-64 (median age at diagnosed is 62)[5].

The percent of female breast cancer deaths is highest among women aged 55-64 (median age at death is 68). Women who are diagnosed at an advanced age may be more likely than younger women to die of the disease. Five-year relative survival depends on the stage at which the cancer is diagnosed. There are better chances of surviving five years after being diagnosed when the breast cancer is detected earlier. Five-year relative survival is 99% for localized stage, 85% for regional stage, and 27% for distant stage.

ii. Classification

Breast cancer is a complex disease with multiple subtypes, each characterized by distinct morphology, molecular profile, clinical behavior and treatment options. The majority of breast cancer tumors (95%) are derived from the inner lining epithelium of the ducts or the lobules of the breast. Breast cancer originating from the ducts are known as ductal carcinomas, while those originating from the lobules are known as lobular carcinomas. The most common type of breast cancer is ductal carcinoma. Ductal and lobular carcinomas are divided into two types based on their histological appearance: 1. in situ, noninvasive neoplastic cells that are limited to the

epithelial layer; 2. invasive carcinoma, which denotes a cancer that has moved through the basal lamina of the epithelial layer and has invaded the surrounding stroma. Ductal invasive carcinoma is the most common form of breast cancer and represents 80% of all breast cancer diagnoses[6].

Breast cancers are classified into three clinical subgroups based on similarities in the gene expression profile and molecular classification. The most common subtype accounts for 70-80% of all the cases is estrogen/progesterone receptor positive (ER+/PR+) known as luminal breast cancer. The second subtype is human epidermal growth factor 2 positive (HER2+ overexpressed and amplified), which accounts for 15% of all the cases. Both luminal and HER2+ respond to hormone and antibody therapy respectively. The third subtype is the basal-like or also known as triple-negative (ER-, PR-, HER2-), which accounts for 15% of all the cases and responds to chemotherapy and polymerase inhibitors[7].

III. EPITHELIAL-MESENCHYMAL TRANSITION

i. Definition and Classification

Epithelial cells are polarized cells with strong adherent cell-cell and cell-basal lamina junctions. The basement membrane separates the epithelial lining from the stroma or connective tissue. In contrast, mesenchymal cells display fibroblast-like morphology, lack of cell-cell adhesions and have the ability to migrate and invade adjacent tissues. The process by which epithelial cells de-differentiate into mesenchymal cells is called epithelial-mesenchymal transition (EMT). EMT is a biological process that involves a set of multiple and dynamic transitional states between epithelial and mesenchymal phenotypes with a reverse process called mesenchymal-epithelial transition (MET)[8].

EMT programs have been classified into 3 types; type 1 relates to embryo formation and development; type 2 is related to tissue regeneration, organ fibrosis and wound healing; and type 3 is associated with cancer progression and metastasis[8,9].

Type 3 EMT can be activated in epithelial cancer cells, causing them to downregulate adhesion junctions, acquire invasive properties and develop metastatic growth characteristics. Epithelial cancer cells that undergo full or partial EMT participate in cancer progression including invasion through the basement membrane, migration through stroma and intravasation followed by transport through the lymphatics or circulation**.** In addition, EMT confers a stem cell phenotype and resistance to cell death, immunotherapy and chemotherapy[8,10].

ii. EMT-mediated changes in gene expression

During EMT, cancer cells oscillate between intermediary phases of epithelial and mesenchymal phenotypes[8]. Epithelial cancer cells that undergo complete EMT suffer complex cellular and molecular alterations such as changes in morphology, acquisition of motility and changes in gene expression profile that ultimately promote invasiveness and metastasis. These events involve reduction in epithelial proteins like cell-cell adhesion molecule E-cadherin, accompanied by the upregulation of mesenchymal proteins such as Vimentin, N-cadherin, fibronectin and α-smooth muscle actin which facilitate migration and invasion[8,11].

iii. EMT-inducing transcription factors

EMT is driven by several transcription factors including zinc-finger binding transcription factors SNAIL1and SNAIL2; zinc finger E-box–binding homeobox ZEB1 and ZEB2; and basic-loop-helix Twist in respond to a microenvironmental stimuli[12]. These EMT-inducing transcription factors repress expression of genes associated with cell-cell adhesion, while activating genes associated with the mesenchymal phenotype[10]. SNAIL1, SNAIL2 and TWIST repress the

expression of E-cadherin, tight junction proteins (claudin and occludin) and epithelial-specific intermediate filament proteins (cytokeratins)[10,11]. These factors activate the expression of fibronectin, N-cadherin and genes encoding extracellular matrix proteins (collagen and matrix metalloproteinases - MMPs) and drive cancer stem cell features[11]. ZEB factors are known to repress directly E-cadherin and the tight junction associated signaling proteins ZO-1, and activate N-cadherin and MMPs[10,11].

iv. EMT-inducing signaling pathways

EMT is induced in response to stimuli from the tumor microenvironment, like growth factors, cytokines, hypoxia, and contact with the surrounding extracellular matrix (ECM)[11]. These stimuli activate signaling pathways including transforming growth factor-β (TGF-β), Notch, WNT/β-catenin, Hedgehog and receptor tyrosine kinases which in turn activate EMT-transcription factors to initiate the EMT process[11,12].

TGFβ is the most well-characterized signaling pathway that induces EMT by acting through SMAD-mediated and non-SMAD signaling[11,12]. SMAD-mediated signaling activates SMAD factors (SMAD 2/3/4), resulting in an activated complex that interacts with several transcription factors (SNAIL1, SNAIL2 and TWIST) to regulate genes involved in the EMT process. In addition, SMADs directly activate the expression of some mesenchymal genes encoding fibronectin, vimentin and collagen. TGFβ also induces non-SMAD signaling pathways like NF-κB, Par6, small GTPases, PI3K/AKT/TOR and MAP kinase that regulate cytoskeleton organization, cell survival, migration and invasion contributing to the EMT process[13].

Receptor tyrosine kinases are activated by several growth factors including epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF), leading to the RAS–RAF–MEK–ERK MAPK signaling

cascade to induce EMT. On the other hand, Notch, WNT/β-catenin and Hedgehog pathways promote downregulation of E-cadherin levels[11,13].

Hypoxia is as an example of a major external stimulus that leads to changes in mitochondrial function, leading to the upregulation of hypoxia-inducible factor 1-α (HIF1α), hepatocyte growth factor (HGF), SNAI1 and TWIST1 and activation of the Notch or NF-κB pathways[12,13]. Inflammation also promotes EMT, involving cytokines such as interleukin-6 and TNF-α[11]. Additionally, TNF-α promotes cancer invasion, angiogenesis and stemness properties associated with EMT programs[11–13]. The pathways that induce EMT also involve microRNAs (miRNAs)[11,12]. Specific miRNAs target mRNAs encoding adhesion junction and polarity complex proteins and signaling mediators. For example, miR-194 represses N-cadherin expression, preventing cell migration and invasion. The miR-200 family suppresses the EMT process by targeting ZEB1/ZEB2 and maintaining E-cadherin expression[12].

IV. Metabolic Reprogramming

Cancer cells undergo metabolic alterations to adapt to the tumor microenvironment. Collectively these alterations are referred as metabolic reprogramming, which confers growth and/or survival advantages on cancer cells[14]. Metabolic reprogramming of cancer cells is associated with increased nutrient uptake including glucose and glutamine, along with alterations of intracellular metabolic pathways like glycolysis and tricarboxylic acid (TCA) cycle. In some cases, metabolic reprogramming allows cancer cells to accumulate precursor molecules needed during rapid cell proliferation, and to produce ATP as well as antioxidant metabolites, such as NADPH. In addition, metabolic reprogramming promotes access to diverse metabolites that serve as cofactors or substrates for enzymes involved in signaling pathways and gene regulation[15]. Finally, cancer cells that undergo metabolic reprogramming are able to alter the composition of their tumor

microenvironment, affecting the behavior of neighboring cells like tumor-associated fibroblasts, endothelial cells, and innate and adaptive immune system cells to favor tumor growth and dissemination[14,16].

i. Mitochondrial Respiration

Most differentiated and nonproliferating cells metabolize glucose into pyruvate via glycolysis, and then most of that pyruvate enters into mitochondria where it is converted to acetyl-CoA, which is completely oxidized to carbon dioxide (CO_2) in the tricarboxylic acid (TCA) cycle. The electrons released from this oxidation process are transferred to NAD+ and FAD+, to generate 3 NADH and one $FADH_2$. These reduced cofactors are used to create a proton gradient across the mitochondrial membrane by a pathway called the electron transport chain (ETC). The energy stored in this gradient is used to drive ATP synthesis by a process called oxidative phosphorylation (OXPHOS). In OXPHOS, oxygen must be present to receive electrons from the protein complexes as it is the final electron acceptor**.** A maximum of 32 molecules of ATP (2 from the TCA cycle and 30 from OXPHOS) are generated per two molecules of acetyl-CoA entered into the TCA cycle.

In a tumor there are regions with different nutrient and oxygen concentrations, driving selection for those cancer cells that are able to adapt their metabolism to changing external factors. Relatively quiescent cancer cells that reside within an oxygenated tumor microenvironment utilize glucose preferentially for mitochondrial acetyl-CoA generation, which is then subjected to oxidation in the TCA cycle and later OXPHOS for ATP production[14]. In fact, studies in breast and cervical cancer cells showed that mitochondrial OXPHOs contributes more than 70% and 90% respectively in overall ATP generation under normal conditions[17]. On the other hand, aggressive and rapidly proliferating cancer cells switch to a glycolytic metabolism to generate ATP regardless

of the presence of oxygen, a phenomenon referred to as "the Warburg effect" or aerobic glycolysis[18].

ii. Aerobic glycolysis or "Warburg effect"

In the 1920s, the German physiologist Otto Warburg made an observation regarding tumor metabolism. He showed that tumor tissue metabolizes more glucose compared to nonproliferating normal tissue resulting in high lactate production even in the presence of oxygen. This observation has come to be known as the "Warburg effect" or aerobic glycolysis. Most recently, studies have shown that proliferating cancer cells exhibit increased glucose uptake and glycolytic flux[14]. Most of the pyruvate from glycolysis is converted to lactate rather than be transported into the mitochondria to maintain oxidative phosphorylation. Although glycolysis has a low efficiency to produce ATP (2 molecules per glucose), the rate of ATP synthesis is faster compared to OXPHOS. Consequently, by increasing glucose uptake and glycolytic flux, the amount of ATP produced from glycolysis can surpass the amount produced from OXHPHOS. Additionally, glucose catabolism provides metabolic intermediates needed for rapid cell growth, and reduces oxygen dependency and reactive oxygen species (ROS) production. Furthermore, lactate generated by glycolytic cancer cells acidifies the extracellular microenvironment which facilitates tumor cell migration and invasion[19]**.** Recent studies have shown that the Warburg effect phenomenon is typical in proliferating tumor cells and also in some stromal cells like cancer-associated fibroblasts (CAFs)[14,18].

iii. Reverse glycolysis or "Reverse Warburg effect"

In a single tumor there are numerous heterogeneous cell populations. Thus, both mitochondrial respiration and aerobic glycolysis contribute differently to each population and favor tumor growth and survival under different conditions. Cancer cells have flexible metabolic phenotypes. Under

nutrient/glucose-limited conditions, glycolytic cancer cells can alter metabolism, via activation of signaling pathways and transcription factors, and restore partly suppressed OXPHOS[20,21]. Those cancer cells able to perform oxidative metabolism and particularly located in close proximity to blood vessels, can utilize energy-rich fuels (such as pyruvate, ketone bodies, fatty acids and lactate) produced by their neighboring cells (proliferating cancer cells and CAFs) to fuel mitochondrial OXPHOs for ATP production[22]. This utilization of lactate and other energy-rich fuels by cancer cells capable of using oxidative metabolism is referred to as the "Reverse Warburg effect"[23]. Tumor cancer cells affect their tumor microenvironment to maintain "Reverse Warburg effect". For example, tumor cells have been shown to secrete hydrogen peroxide, which causes oxidative stress in neighboring CAFs. This induces aerobic glycolysis accompanied with high lactate production in CAFs, and the lactate in turn fuels OXPHOS in oxidative cancer cells[24].

V. Lactate Metabolism

i. Lactate metabolism key players

Highly glycolytic cancer cells produce high amounts of pyruvate that is converted into lactate by tetrameric isozymes called lactate dehydrogenases (LDHs). These isozymes are formed by the combination of two different subunits, LDHA and LDHB. The subunit LDHA has a higher affinity for pyruvate and a higher Vmax for pyruvate reduction than LDHB. As a result, the tetramer with more LDHA subunits catabolizes pyruvate to lactate and produces NAD+, which is essential for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in glycolysis. By contrast, tetramers rich in the LDHB isoform convert lactate to pyruvate, which allows cells to use lactate as a nutrient source for oxidative metabolism and/or for gluconeogenesis/reverse glycolysis. LDHB is ubiquitously expressed, especially in oxidative cardiac muscle, whereas LDHA is the predominant isoform found in Type II skeletal muscle fibers and other highly glycolytic tissues[25].

Lactate levels within the extracellular tumor microenvironment are determined by the balance of lactate uptake and lactate export by tumor and tumor-associated cells. Lactate is transported across the plasma membrane of cancer cells through monocarboxylate transporters (MCTs). MCTs are proton-linked transporters and possess different affinities for lactate, which, in turn, partly determines the direction of lactate flux. Thus, secretion of lactate is through the low-affinity lactate transporter MCT4 (Km≈28 mmol/L) and lactate uptake primarily occurs through the high-affinity MCT1 transporter (Km≈1– 3.5 mmol/L)[26]. In normal physiology, MCT4 is highly expressed in glycolytic tissues like type IIB skeletal muscle fibers and astrocytes, whereas MCT1 is highly expressed in oxidative tissues, including cardiac muscle, Type I skeletal muscle fibers, and neurons.

ii. Lactate as a nutrient source

More than a waste product of glycolysis, lactate was recently identified as a major energy fuel for oxidative tumor cells. Lactate, secreted by glycolytic tumor cells through the lactate exporter, MCT4, may be taken up by oxidative tumor cells through lactate importer MCT1, converted into pyruvate by LDHB and used as a fuel for oxidative metabolism[27,28]. Thus, oxidative tumor cells spare glucose which may, in turn, reach glycolytic tumor cells. This "lactate shuttle" is part of the metabolic symbiosis between tumor cells and it is an important component in the "Reverse Warburg effect" phenomenon described in different types of cancer including breast cancer [27–30].

iii. Lactate signaling molecule

Lactate plays a critical role in cancer progression and metastasis by promoting cancer cell migration, immune escape and angiogenesis [31–33]. Extracellular lactate promotes tumor cell migration and angiogenesis by inducing the secretion of the cytokine IL-8 and the vascular endothelial growth factor (VEGF) by endothelial cells [31,34,35]. Moreover, lactate is able to block

immune surveillance by inhibiting the differentiation of monocytes and natural killer cells[33,36]. Also, the secretion of lactate through MCTs releases H+, decreasing the extracellular pH that affects T-cell function and provokes local inflammation[37,38].

Recently two signaling pathways have been identified where lactate acts as a "signaling molecule" to promote angiogenesis and tumor growth. First, lactate inhibits prolylhydroxylase 2 (PHD2), the enzyme that hydroxylates HIF1α, inhibiting its ubiquitination and subsequent proteasomal degradation. Thus, lactate promotes angiogenesis and other HIF1α-related effects in the absence of hypoxia[39]. Second, lactate also inhibits the proteosomal degradation of the tumor promoter, N-Myc Downstream Regulated Gene 3 (NDRG3), another target of PHD2. Also, lactate binds directly to NDRG3 activating the Raf-ERK pathway to promote angiogenesis and cell growth during hypoxia[40].

iv. GPR81

G protein-coupled receptor-81 (GPR81) has been identified as the endogenous receptor for lactate. Lactate activates GPR81 with a EC50 value of 5 mM, which is within the physiologic concentration range (1 to 20 mmol/L) of lactate within breast tumors. Also known as Hydroxy Carboxylic Acid Receptor 1 (HCAR1), GPR81 is part of the HCAR family that consists of three highly homologous receptors; GPR81 (HCAR1), GPR109a (HCAR2) and GPR109b (HCAR3), which are regulated by the specific agonist, L-lactate, 3-hydroxybutyrate and 3-hydroxyoctanoate, respectively[41]. In normal cell types (e.g., adipocytes), GPR81 is coupled to Gi/q, and activation of the receptor by L-lactate results in decreased cellular levels of cAMP and increased cellular levels of Ca^{2+}. This receptor has been mainly studied in adipocytes, in which extracellular lactate specifically activates GPR81 to decrease the cAMP production and ultimately reduce lipolysis[42]. GPR81 is receiving increasing attention in the cancer field as a result of its upregulated expression

in several types of cancers like pancreas, colon, liver, breast, lung and cervix which in many cases the correlates with tumor growth, chemoresistance and metastasis [43–46]. In particular, one recent study showed that GPR81 regulates lactate transporters MCT1 and MCT4 in pancreatic cancer cells and promotes cell growth/survival under nutrient stress conditions [44].

VI. Significance

Although, there is an increasing interest in the lactate metabolism of cancer, there are few studies on lactate metabolism in the context of metastatic transformation. Since EMT induces metastatic transformation, in chapter 3 we studied the metabolic reprogramming induced by EMT in breast cancer. We analyzed the alterations in the lactate metabolism of two epithelial breast cancer cell lines and their respective post-EMT mesenchymal breast cancer cell lines. In addition, we studied the role of the lactate importer MCT1 in the epithelial breast cancer cells.

The lactate receptor GPR81 has been shown to promote cancer progression. In chapter 4, we studied the expression of GPR81 in human breast cancer tissues and in a EMT breast cellular model. We also studied the role of this receptor in epithelial breast cancer cells and its potential as a therapeutic target.

CHAPTER 2

MATERIALS AND METHODS

Cell culture

BT-474 and MCF-7 cell lines were obtained from the American Type Culture Collection (Manassas, VA). EMT in MCF-7 and BT-747 cells was induced using prolonged mammosphere culture method as reported previously[47,48]. BT-474 and MCF-7 and their corresponding EMT-derived BT-474-M and MCF-7-M cells were grown in DMEM/F-12 containing 10% heat inactivated FBS (Gibco, Grand Island, NY) and 1× MycoZap™ Plus-CL antibiotic (Lonza,Walkersville, MD). BT-474 and MCF-7 cells were additionally supplemented with 1× Insulin–Transferrin–Selenium solution (Gibco, Grand Island, NY). All cells were incubated at 5% CO2 and at 37 °C.

Physiological modified medium and drugs

RPMI 1640 Medium Modified without l-Glutamine, without Amino acids and Glucose (US Biological, Swampscott, MA) was supplemented with 5 mM D-glucose, 2.5 mM L-glutamine (Sigma, St. Louis, MO), 1× MEM essential and non-essential amino acid mixtures, 10% heat inactivated FBS, 15 mM HEPES (Gibco, Grand Island, NY); 0.5 mM sodium pyruvate, chemically defined lipid mixture 1, 15 mM sodium bicarbonate, 1 mM Na-lactate (Sigma, St. Louis, MO) and 1× MycoZap™ Plus-CL antibiotic (Lonza,Walkersville, MD).

RPMI-1640 Medium Modified with L-glutamine, without phenol red and sodium bicarbonate (Sigma, St. Louis, MO) was used in assays where cells were treated with β-Estradiol or Tamoxifen. MCT1 inhibitor SR 13800, β-Estradiol and Tamoxifen (Tocris Bioscience, Bristol, UK) solutions were made using ethanol and were used at concentrations of 100 nM, 10 nM and 1 μM, respectively.

3-dimensional culture

Corning™ Matrigel™ GFR (Fisher Scientiic) or Matrigel™ (Corning) was used as culture matrix following a modified version of the previously described protocol for 3-dimensional on-top assay[49]. Twenty-four-well plates were coated with 200ul of the mix of 1:1 Matrigel™/Physiological modified medium supplemented with 1x Insulin–Transferrin–Selenium solution (Gibco, Grand Island, NY) and kept at 37°C for 30 minutes to allow the Matrigel™ to solidify. All cell lines were suspended in physiological modified medium supplemented with 1x insulin and seeded into the coated twenty-four-plates. Epithelial and mesenchymal cells were seeded in 250µl at 100, 000 cells/well and 20, 000 cells/well, respectively, and incubated at 37°C for 30 minutes to allow cell attachment to the Matrigel. All wells were overlaid with 250µl of physiological modified medium containing 4% Matrigel™. Culture medium was replaced with fresh medium daily and cells were used for each assay after seven days, unless otherwise indicated.

Imaging of 3-dimensional structures

Cells were cultured in 3-dimensional Matrigel and glass bottom 24-well plate (MatTek Corporation, MA) for seven days. Phase contrast images of the 3-dimensional structures of epithelial and mesenchymal cells were imaged using a Zeiss Axio Observer inverted microscope (Carl Zeiss MicroImaging Inc., Thomwood, NY) equipped a QImaging Retiga EXi CCD digital camera (QImaging, Surrey, BC). For immunofluorescence images cell lines grown in 3-dimensional Matrigel at the seventh day were fixed in 2% formaldehyde in 1X PBS for 20 minutes. Cells were washed with 1X PBS and then treated with 0.1% Triton-X 100 for 10 minutes. Cell were washed with 1X PBS and blocked with 1% bovine serum albumin (BSA) for 20 minutes. After blocking, cells were incubated with F-actin-staining phalloidin staining solution for 20 minutes at room temperature. Phallotoxin staining solution was prepared with 5µl Alexa-488

Phallotoxin (Invitrogen, Carlsbad, CA) stock in 200µl 1X PBS for each well to be stained. SYTOX^{TM} orange dye (Invitrogen, Carlsbad, CA) was used to stain nuclei. Images were captured using a Zeiss Pascal confocal system with a 40X 1.2 NA objective (Carl Zeiss Microscopy, Narashige, MN).

Transient transfection of siRNA

MCF-7 cells were seeded in 250ul at a density of 150, 000 cells/well in twenty-four-well plates coated with 200ul of the mix of 1:1 Matrigel™/Physiological modified medium supplemented with 1x Insulin–Transferrin–Selenium solution (Gibco, Grand Island, NY) and transiently transfected with Trilencer-27 human siRNA (siNT, siGPR81 "B" and siGATA3 "A") at a final concentration of 10 nM (Origene, Rockville, MD) with Lipofectamine™ RNAiMAX transfection reagent (Thermo Scientific, Rockford, IL). After 8 hours of transfection cells were overlaid with 250µl of physiological modified medium containing 4% Matrigel™. Culture medium was replaced with fresh medium every day and cells were used for each assay after 72 or 96 hours after transfection.

Growth and viability assays

For a seven-day growth curve, epithelial and mesenchymal cells were plated at a density of 100,000 cells/well and 20,000 cells/well, respectively in 3-dimensional Matrigel™ culture. MCF-7 cells were seeded at a density of 150, 000 cells/well for transient transfection and treatments with MCT1 inhibitor SR 13800 or Tamoxifen (Tocris Bioscience, Bristol, UK). Cells were recovered from Matrigel by incubating them with 5 mM EDTA (Gibco, Grand Island, NY) in ice cold 1x PBS for 30 minutes at 4°C and centrifuged for 5 minutes at 1000 rpm. Recovered cells were digested with 0.25% trypsin for 4 min and resuspended in DMEM with 10% FBS. The cell number

was calculated on hemocytometer at specific time point. Cell number viability assays were performed three times for each cell group.

Real-time qPCR and primers

Total RNA was isolated from cell lines using TRIzol Reagent (Ambion RNA, Carlsbad, CA), following the manufacturer's instruction. cDNA was synthesized 1 μg of total RNA using iSCRIPT cDNA synthesis kit (Bio-Rad, Hercules, CA). SYBR green based SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA) was utilized to perform real-time PCR from 50ng of cDNA. Gene expression was normalized to TATA-box binding protein 1 (TBP1) using $2^{-\Delta Ct}$ method. Specificity of the primer sets was confirmed by melting curve analysis. Primer sequences were listed in Appendix section.

cDNA Arrays

Real-time qPCR was performed in two TissueScan™ breast cancer and normal tissue cDNA arrays I and II (Origene, Rockville, MD). Gene expression was normalized to normal tissue using $2^{-\Delta Ct}$ method.

Real-time metabolic analysis

Simultaneous multiparameter metabolic analysis of all four cell lines in culture was performed in the Seahorse XF96 extracellular flux analyzer (Seahorse Bioscience, Billerica, MA). Epithelial and mesenchymal cells were seeded in Seahorse 96-well XF cell culture microplate at 15,000 cells/well and 8,000 cells/well, respectively, and pre-incubated at 37°C in 5% CO_2. Following 48 hours of incubation, the culture medium was replaced with assay media (unbuffered Seahorse XF Base medium supplemented with 5 mM D-glucose, 0.5 mM L-glutamine, 0.1 mM sodium pyruvate and 1 mM Na-lactate, pH 7.4) one hour before the assay in a non-CO_2 incubator and for the duration of the experiment. Mitochondrial complex inhibitors (1 μM oligomycin, 1 μM FCCP, 0.5

μM rotenone) were preloaded in the injection ports. Three baseline measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were made and were averaged to give the 100% starting value. After establishing the OCR and ECAR baselines, mitochondrial complex inhibitors (oligomycin, FCCP, rotenone) were injected consequently and after a short period of mixing, OCR and ECAR measurements were made using photodetectors with specific excitation and emission wavelengths of oxygen (532/650 nm) and protons (470/530 nm; 28). These experiments were performed two times, each with ten samples per cell type. The data were normalized by cell number.

Glucose uptake and lactate production

Epithelial and mesenchymal cells were plated at a density 100,000 cells/well and 20, 000 cells/well in 3-dimensional Matrigel™ culture for seven days. For transient transfection, MCF-7 cells were seeded at a density of 150, 000 cells/well and medium was changed daily. Glucose and lactate concentrations in the culture medium were determined by fluorometric-based Glucose Assay Kit and Lactate Assay Kit (BioVision, Inc., Milpitas, CA) according to the vendor's instructions using Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT). The amount consumed or produced by cells was determined by comparing the concentration in the medium incubated without cells and then normalized to cell number. These assays were performed three times with two experimental replicates for each cell group.

Western blot and antibodies

Cells were collected form 3-dimensiona Matrigel™ culture (as described previously) and lysed with RIPA buffer (TEKnova, Hollister, CA) containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, IL). The lysates were clarified by centrifugation at 4 °C for 10 min at 12,000 ×g. The concentration of protein in the supernatants was measured using BCA

assay (Thermo Scientific Pierce, Rockford, IL). Total proteins (20 μg/lane) were separated on a 10% SDS-PAGE and were transferred to nitrocellulose membrane. The membranes were blocked with 5% BSA in 1× TBST (β-actin, LDHA and LDHB) or with 5% non-fat milk (MCT1, MCT4 and Cleaved PARP) and subsequently incubated with primary and secondary antibodies according to the vendor's instructions. Blots were visualized by using Amersham ECL prime (GE Healthcare Life Sciences, Buckinghamshire, UK) and imaged using G:box imaging system (SynGene, Cambridge, UK). Primary antibodies to LDHA (1:10,000) was purchased from Cell Signaling Technology (Danvers, MA); Cleaved PARP (1:1000 from Cell Signaling Technology (Danvers, MA) was a gift by Dr. K. Claffey, Dept of Cell Biology and Center for Vascular Biology, UConn Health); β-actin (1:2500) and MCT1 (1:1000) were purchased from Abcam (Cambridge, MA); LDHB (1: 10,000) was purchased from OriGene (Rockville, MD). MCT4 (1:1000) was purchased from Millipore (Billerica, MA). All images were imported to Image J for pixel grayscale intensity analysis. Western blot analysis was performed three times using two independent protein samples for each cell group.

Nuclear Magnetic Resonance (NMR) Spectroscopy and Data Analysis

Cells were collected form 3-dimensional Matrigel™ culture (as described previously) and lysed with 1:1:1 methanol/chloroform/water in sequence with 5 seconds of gyration between additions. In order to optimize chemical extraction, non-trypsin-based methods were employed as follows[50]. A 10 μL sample was then removed for cell counting. Resulting lysed solution was then allowed to separate overnight at 4°C to form resultant aqueous and organic layers. Aqueous layer containing metabolites of concern were then manually extracted and resulting solution was subjected to addition of Chelex (approx. 3mg/mL) for removal of divalent ions, which was then removed through vacuum filtration using Buchner filter. Solution was then lyophilized overnight to form

solid components. 800 µL of D_2O with 10 mM EDTA and 1 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) was added and solution was centrifuged at 3000RPM for 5 minutes prior to placement in standard 5 mm NMR tube for ^1H NMR.

^1H NMR experiments were performed on 500 MHz Agilent spectrometer with HCN cold probe and a VNMRS console. Default pre-saturation pulse sequences were utilized with the following parameters: pulse-width = 7.2 µs, sweep-width = 8389.26 Hz, 2460 complex points, D1 = 1.5 s, SATPWR = 6 dB on H_2O signal, and 64 transients. Resulting ^1H NMR underwent Fourier Transformation and data was further analyzed in MestReNova NMR software, version 9.0.0. Processing template for all spectra had the following time domain corrections: drift correction to tail points of 5% and apodization through an exponential of 1.5 Hz. The frequency domain had global phase correction and a baseline correction using Bernstein polynomial of n=3. Reference signal was set as the singlet produced off of DSS at 0 ppm. Lactate and Pyruvate were identified on the spectra through prior titration analyses and the absolute area under the curve was used for their methyl groups with respect to methyl groups found off of DSS with the usage of Line-Shape analysis. These areas were used to calculate concentrations by a ratio of the absolute area to the number of protons in the signal used. Resulting concentrations in millimolar were then exported into an Excel spread sheet containing cellular counts to determine a normalized concentration per number of cells.

Statistical analysis

Ordinary one-way ANOVA followed by Sidak's multiple comparison post-test or two-way ANOVA was performed to determine statistical significance. $P < 0.05$ was considered to be significant. Statistical analysis was performed using GraphPad Prism software (La Jolla, CA).

CHAPTER 3

EPITHELIAL-MESENCHYMAL TRANSITION INDUCES CHANGES IN THE LACTATE METABOLISM OF BREAST CANCER CELL LINES

I. Abstract

Lactate is both a metabolite of glycolysis and a component of several signaling pathways. Although recent studies indicate that lactate is a critical regulator of cancer development, very little is known about lactate metabolism in the context of metastatic cancer. Epithelial-mesenchymal transition (EMT) promotes metastasis by inducing invasive properties in epithelial tumors. To determine whether EMT induces metabolic alterations, we have studied two epithelial breast cancer cell lines (MCF-7 and BT-474) and their respective EMT-induced mesenchymal progeny (MCF-7 M and BT-474 M) for changes in lactate metabolism. Metabolic analysis revealed that EMT induced an enhanced glycolytic profile in mesenchymal breast cancer cells along with the overexpression of glucose transporters (GLUT3, GLUT12), lactate dehydrogenases (LDHA, LDHB) and monocarboxylate transporter 4 (MCT4). In contrast, epithelial breast cancer cells preferentially used oxidative phosphorylation (OXPHOS) to produce ATP. These aerobic epithelial breast cancer cells highly expressed the estrogen receptor α (ERα) and the monocarboxylate transporter 1 (MCT1). We found that the epithelial breast cancer cells but not the mesenchymal breast cancer cells imported lactate through MCT1 and used it as an alternative nutrient to support cell growth. The strong expression of MCT1 in epithelial breast cancer cells was regulated by ERα signaling pathway and the transcription factor GATA3. In addition, the co-treatment of epithelial breast cancer cells with Tamoxifen and MCT1 inhibitor inhibited cell proliferation to a significantly greater extent than with either drug by itself. Our study suggests the potential of MCT1 as a therapeutic target to treat ER-positive epithelial breast cancer.

II. Introduction

Invasive breast cancer (IBC) is the predominant form of cancer among women worldwide and the second cause of cancer-related deaths in women in the United States[51 5]. The most common type of IBC, called Luminal A, is characterized by an adhesive epithelial phenotype and overexpression of the estrogen receptor α (ERα). Although Luminal A IBC is a relatively indolent, non-aggressive form of cancer, failure of early detection is significantly associated with metastatic disease, which in turn is responsible for the majority of deaths in breast cancer patients.

Metastatic dissemination of IBC involves a succession of complex events that ultimately lead to the colonization of distant organs[52]. Either complete or partial epithelial-mesenchymal transition (EMT) has been implicated in the earliest stages of metastasis[8]. EMT allows for the detachment of cells from the main tumor mass, and increases motility and invasiveness of tumor cells, coupled to increased autonomous production of extracellular matrix. Differing degrees and/or modes of EMT are orchestrated by a range of transcriptional and epigenetic mechanisms among the heterogeneous population of cells within a tumor. The process of EMT can be induced by exposure of cancer cells to an altered microenvironment (e.g., hypoxia, increased cytokines) that is imposed by the tumor itself (or a subpopulation thereof), by surrounding cells (e.g. macrophages) in response to a growing tumor, and/or by systemic physiology that has been altered (e.g., by obesity). Additional somatic mutations in highly proliferative cells also likely contribute to a changing microenvironment and/or the responsiveness of tumor cells to the microenvironment [10 12].

Cancer cells frequently exhibit increased glucose uptake, glycolytic rates and lactate production in the presence of oxygen, a process referred to as “aerobic glycolysis” or “Warburg effect” [16]. A large body of recent studies revealed that aerobic glycolysis and other metabolic

changes allow cancer cells to accumulate building blocks for the biosynthesis of macromolecules that are used in anabolic pathways, while simultaneously satisfying the high energy demands and maintaining redox balance needed for cell proliferation[53]. These metabolic changes are collectively referred to as "metabolic reprogramming", now considered a hallmark of cancer cell biology[14,54]. Most studies on metabolic reprogramming have been performed in the context of transformation per se, i.e., the comparison of metabolic pathways in non-neoplastic cells to those in their neoplastic counterparts[55–57]. More recent studies have also demonstrated that metabolic reprogramming occurs within epithelial cancer cells that partially or completely transition to mesenchymal cancer cells. Some studies have indicated that metabolic reprograming contributes to the acquisition and maintenance of the mesenchymal phenotype in breast cancer cells and this cell type exhibits a specific metabolic signature associated with poor clinical outcome in breast cancer patients [58,59].

We previously reported that prolonged 3-dimensional mammosphere culture induced EMT in two distinct epithelial breast cancer cell lines, MCF-7 and BT-474 cells, generating stable populations of mesenchymal cancer cells, termed MCF-7M and BT-474M cells, respectively[47,48]. Using standard 2-dimensional culture on plastic dishes and a standard tissue culture medium (DMEM/F12) containing supra-physiological levels of several nutrients (especially glucose), we characterized these four cell lines with respect to metabolism by assaying the expression of enzymes and specific metabolite transporters associated glycolysis and associated side-pathways, and in terms of glucose uptake and lactate production. These analyses indicated that both epithelial MCF-7 and BT-474 cells are less glycolytic than the two mesenchymal MCF-7M and BT-474M cell lines, but express higher levels of enzymes involved in reverse glycolysis and certain glycolytic side-pathways. In the current study, we directly examined the degree of oxidative vs

glycolytic metabolism in these 4 cell lines, using a culture system that involves both a 3-dimensional Matrigel matrix and media containing the physiological levels of glucose (5 mM), lactate (1 mM), glutamine (0.5 mM) and pyruvate (0.1 mM). In addition, we further examined metabolic differences in lactate flux and utilization in these phenotypically different cell lines. Finally, we showed that MCT1 is required for lactate import and survival of the epithelial, but not mesenchymal, breast cancer cells.

III. Results

Epithelial And Mesenchymal Phenotypes In 3-Dimensional Culture

A major objective of this study was the characterization of aspects of metabolism in the two epithelial MCF-7 and BT-474 cell lines and the corresponding post-EMT mesenchymal MCF-7M and BT-474M progeny within a culture system that was more physiologically-relevant and in which specific nutrients were better defined. Thus, the four cell lines were cultured within a 3-dimensional Matrigel configuration as described by Bissell and colleagues[49]. In addition, the culture medium contained physiological levels of glucose (5 mM), lactate (1 mM), glutamine (0.5 mM) and pyruvate (0.1 mM), with medium changed daily, unless noted otherwise. Note that media was supplemented with 10% FBS, so that final concentrations of these nutrients was moderately higher than the values stated above. Note also that medium was changed daily as opposed to being constantly perfused, so that the levels of these nutrients were subphysiological by the 23rd hour. Under these conditions (termed MPM culture, for Matrigel & Physiological Medium), both epithelial cell lines grew as tightly adherent spheroids for 1 week **(Figure 3.1. A; "E" boxes)**. The two mesenchymal cell lines also formed tight spheroids initially, but after 2 days began invading the Matrigel **(Figure 3.1. A; "M" boxes).**

After 1 week of MPM culture, cells were examined for the expression of specific epithelial, mesenchymal, EMT-related and stem cell-like markers. Epithelial spheroids maintained a high level of expression of the epithelial zonula adherent protein, E-cadherin, and the Luminal A-related transcription factor, GATA3 **(Figure 3.1 B)**. Epithelial cells also expressed relatively high levels of the hormone receptors, ERα, PR and/or HER2 **(Figure 3.1 B)**. In contrast, mesenchymal cells showed no or very low expression of E-cadherin, GATA3 and the three hormone receptors. Mesenchymal cells expressed high levels of the mesenchymal marker, Vimentin, along with EMT-related transcription factors, Zeb 1 and Zeb 2 **(Figure 3.1. B)**. Finally, the two epithelial cell lines displayed high CD24 but low CD44 expression consistent with a mammary cell differentiated phenotype, whereas the two mesenchymal cell lines displayed high CD44 expression and low CD24 expression, consistent with a less differentiated, "breast cancer stem cell" phenotype (Figure 3.1. B). The mesenchymal cells also expressed a mammary stem cell phenotype with high expression of integrin α6 (CD49f) and the loss of the proto-oncogene c-Kit expression[60–62] **(Figure 1B)**. Both mesenchymal MCF-7M and BT-474M cell lines had a higher rate of proliferation, along with elevated expression of mRNAs encoding cyclin B1 and Ki67 **(Figure 3.2. A-B).** These data are consistent with our previous findings in these cells, both in 2-dimensional cell culture and after orthotopic transplantation into mice[47,48].

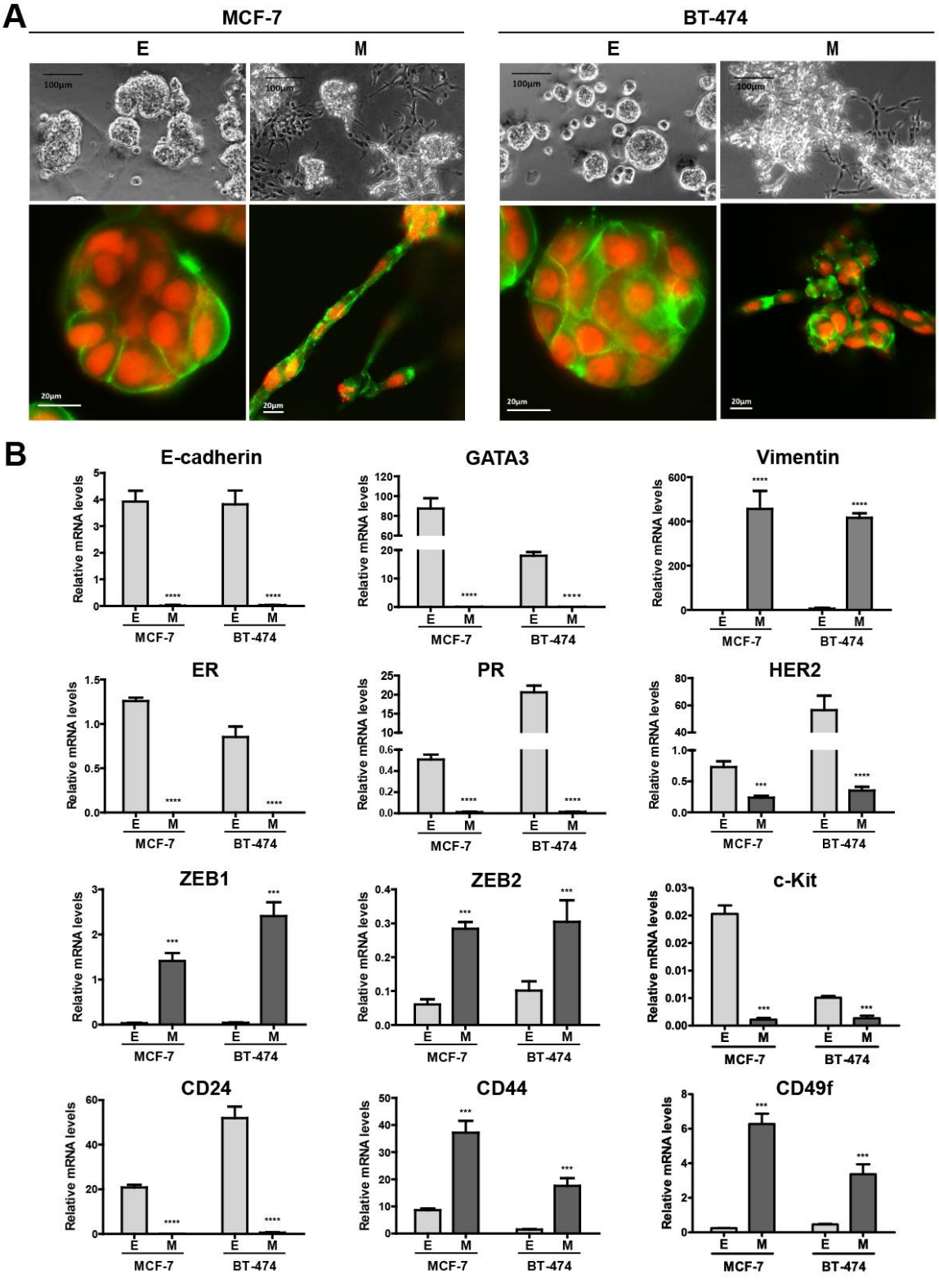

A
MCF-7
BT-474
E
M
100µm
20µm
B
E-cadherin
GATA3
Vimentin
ER
PR
HER2
ZEB1
ZEB2
c-Kit
CD24
CD44
CD49f
Relative mRNA levels
MCF-7
BT-474

Figure 3.1. Epithelial MCF-7 and BT-474 and mesenchymal MCF-7M and BT-474M breast cancer cells in 3-dimensional Matrigel culture.

Top: phase contrast images of epithelial (E) and mesenchymal (M) cells in 3-dimensional Matrigel with physiological modified medium (MPM) for 7 days. Bottom: fluorescence microscopy images of 3-dimensional structures of epithelial and mesenchymal cells. Green: phalloidin staining of F-actin. Red: nuclei counterstained with SYTOX^{TM} orange nucleic acid. Scale bar, 10μm. **B.** Real-time qPCR analysis of epithelial markers, E-cadherin and GATA3; mesenchymal marker, vimentin; hormone/growth factor receptors, ER, PR and HER2; EMT transcription factors, ZEB1 and ZEB2; breast cancer and mammary epithelial stem cell markers, CD24, CD44, CD49f and c-Kit in BT-474, BT-474M, MCF-7and MCF-7M cells. Results are presented as mean ± S.E.M. from three independent experiments (n>6) *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ (ordinary one-way ANOVA).

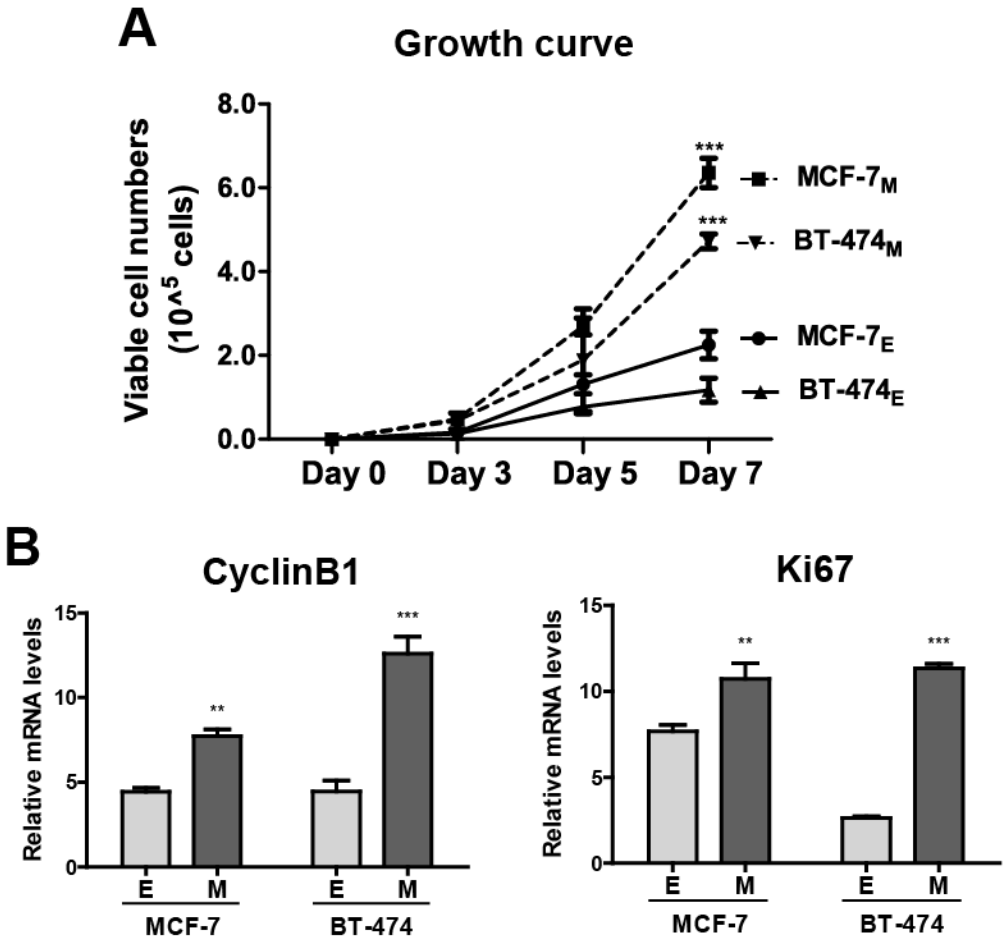


Figure 3.2. EMT-induced mesenchymal MCF-7M and BT-474M breast cancer cells exhibited high cell proliferation rate in 3-dimensional Matrigel culture.

A. Increase in viable cell counts for epithelial (E) and mesenchymal (M) breast cancer cells at days 3, 5 and 7 of culture after subtracting the initial cell number. **B.** Real-time qPCR analysis of proliferation markers: cyclin B1 and Ki67. Results are presented as mean ± S.E.M. from three independent experiments (n=6) *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (two-way ANOVA).

Epithelial MCF-7 And BT-474 Breast Cancer Cells Are More Oxidative, Whereas Post-EMT Mesenchymal MCF-7M And BT-474M Breast Cancer Cells Are More Glycolytic

Subsequent studies investigated whether metabolic reprogramming is linked to EMT as assessed in breast cancer cells culture in physiological medium. Energy metabolism in the two epithelial MCF-7 and BT-474 cell lines and the two post-EMT mesenchymal MCF-7M and BT-474M cell lines was examined in real time by the assay of oxygen consumption rate (OCR; a measure of mitochondrial oxidative phosphorylation), extracellular acidification rate (ECAR; a measure of glycolysis linked to the export of lactate anions and protons) and the dependency on mitochondrial respiration for ATP production (see Chapter 2 Methods & Materials). Epithelial cancer cells exhibited robust basal OCR but a low ECAR, whereas mesenchymal cells consumed oxygen at a lower rate and showed a significantly higher ECAR **(Figure 3.3. A).** We further explored mitochondrial function between epithelial and mesenchymal breast cancer cells by determination of ATP-coupled respiration and maximal respiration capacity. Epithelial cancer cells showed significantly higher mitochondrial ATP production and a greater maximal respiratory capacity than mesenchymal cancer cells **(Figure 3.3. B).** Thus, the metabolism of both post-EMT mesenchymal cell lines was shifted more towards aerobic glycolysis.

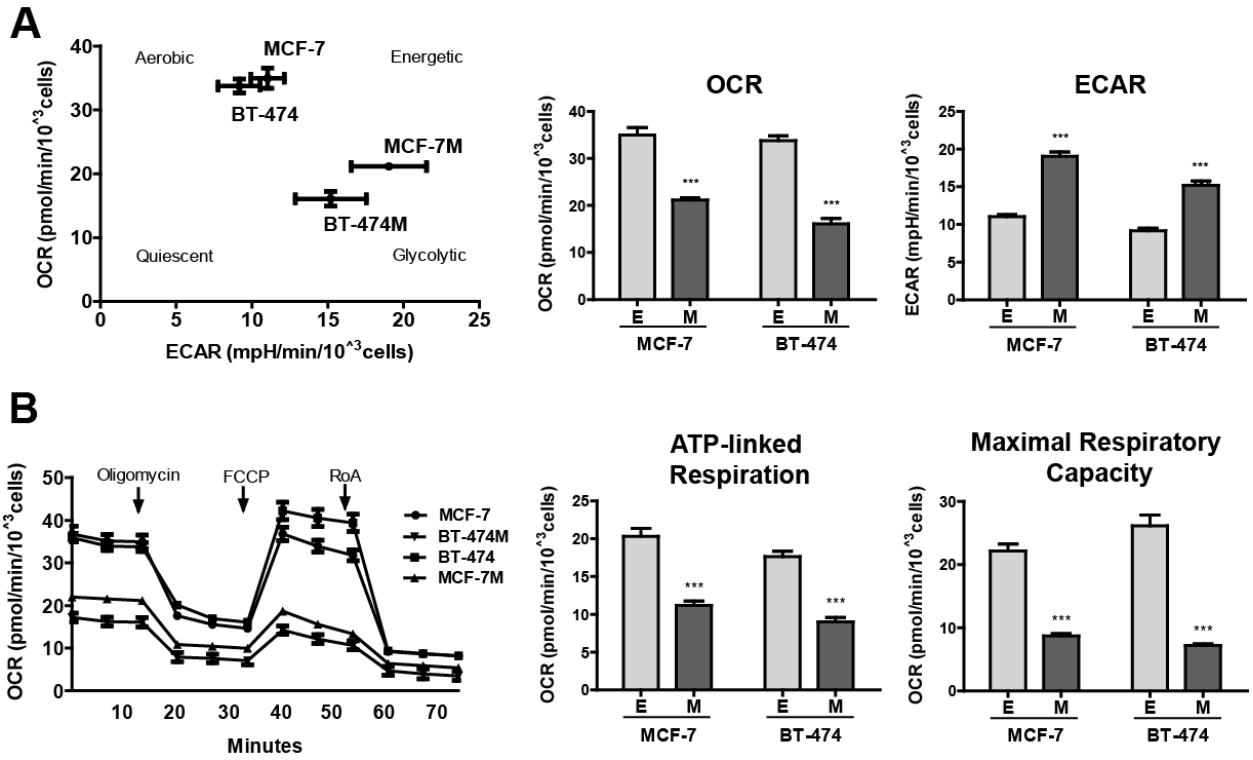


Figure 3.3. EMT-induced BT-474M and MCF-7M mesenchymal breast cancer cells exhibited a more glycolytic profile compared to their parental epithelial MCF-7 and BT-474. Epithelial (E) and mesenchymal (M) cells were seeded on a Cell-Tak-treated XF24 cell culture plate. Data were obtained with the XF Extracellular Flux Analyzer (Seahorse Bioscience). **A.** Metabolic phenotype plot of basal oxygen consumption rate (OCR) vs. basal extracellular acidification rate (ECAR) measurements. **B.** Schematic representation of the mitochondrial function assay. After establishment of baseline OCR, sequential injection of oligomycin (ATP synthase inhibitor), FCCP (mitochondrial uncoupler) and rotenone plus antimycin A (complex I and III inhibitors, respectively) allowed for the determination of ATP-linked Respiration and Maximal Respiratory Capacity. Results are presented as mean ± S.E.M. of two different experiments (n=16) *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (two-way ANOVA).

Efficient Glycolysis and Lactate Export By Mesenchymal Cells

Consistent with a more glycolytic phenotype, both MCF-7M and BT-474M mesenchymal cells displayed elevated glucose uptake **(Figure 3.4. A)**, along with elevated expression of mRNAs encoding GLUT3 and GLUT12, in addition to robust expression of GLUT1 mRNA observed in all 4 cell lines **(Supplemental figure 3.1. A).** Aerobic glycolysis is associated with the reduction of pyruvate to lactate anion coupled to the oxidation of NADH to NAD+ by lactate dehydrogenase A (LDHA)-rich tetramers. The lactate anion and a proton undergo co-transport out of the cell through the monocarboxylate transporters (MCTs; primarily MCT4). The mesenchymal MCF-7M and BT-474M cells exported higher levels of lactate than the epithelial cells **(Figure 3.4. B)**, and expressed high levels of MCT4 mRNA and protein **(Figure 3.4. C-D).** Both LDHA and LDHB protein expression were elevated in the mesenchymal MCF-7M and BT-474M cell lines **(Figure 3.4. E-F)**. NMR analysis revealed that the steady-state levels of intracellular pyruvate and lactate were very low in the two mesenchymal cell lines under MPM conditions **(Figure 3.4. G-I).** Together, these findings demonstrate that both of the post-EMT mesenchymal cell lines rely more heavily on the rapid metabolism of glucose through glycolysis, followed by efficient conversion of pyruvate to lactate and the export of lactate.

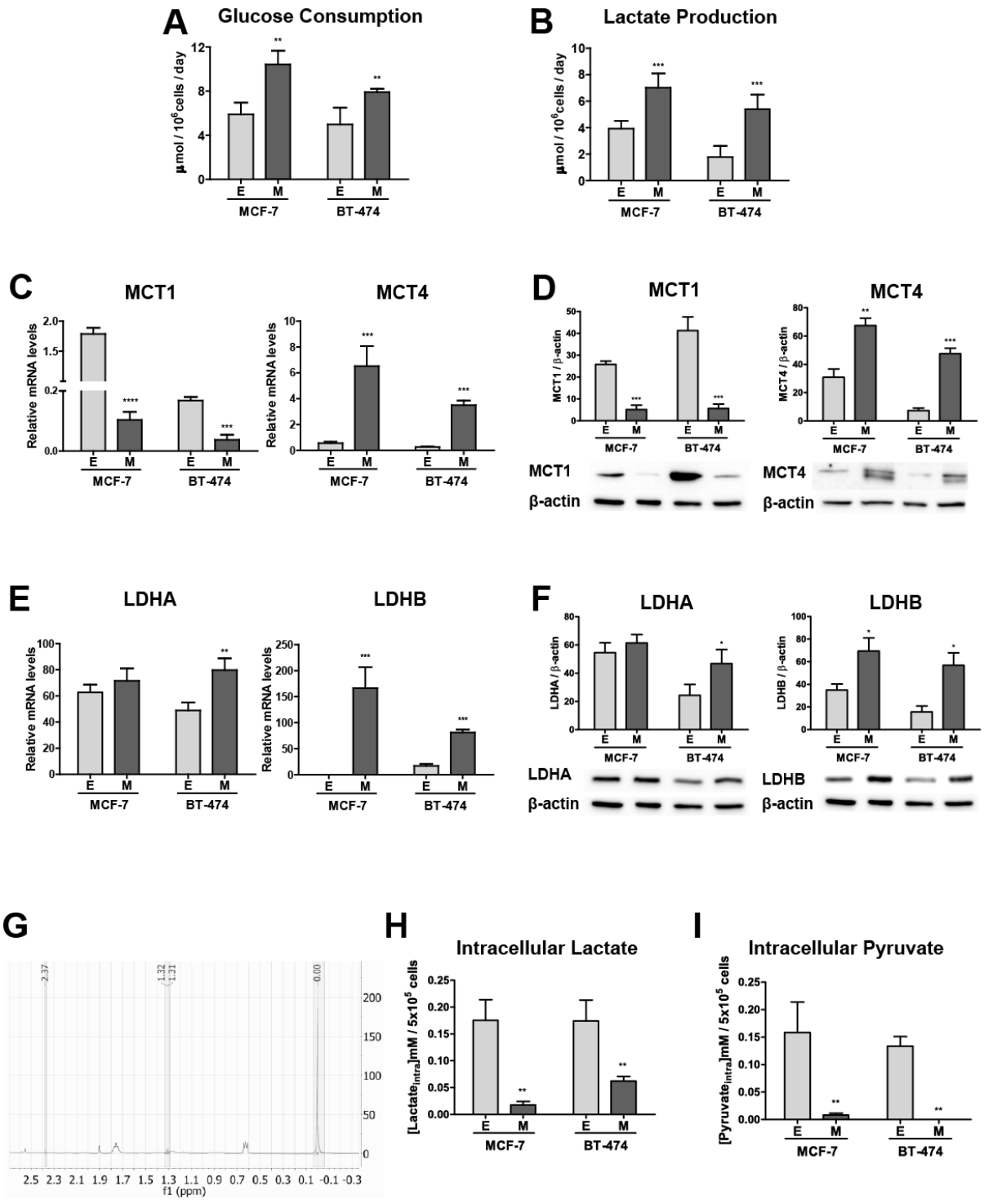

A
Glucose Consumption
µmol / 10^6 cells / day
E
M
MCF-7
BT-474
B
Lactate Production
µmol / 10^6 cells / day
C
MCT1
MCT4
Relative mRNA levels
D
MCT1
MCT4
MCT1 / β-actin
MCT4 / β-actin
β-actin
E
LDHA
LDHB
F
LDHA
LDHB
LDHA / β-actin
LDHB / β-actin
G
f1 (ppm)
H
Intracellular Lactate
[Lactate_intra]mM / 5x10^5 cells
I
Intracellular Pyruvate
[Pyruvate_intra]mM / 5x10^5 cells

Figure 3.4. EMT-induced BT-474M and MCF-7M mesenchymal breast cancer cells exhibited increased lactate production along with upregulation of lactate dehydrogenases and downregulation of specific lactate transporter.

A-B. Glucose consumption and lactate production rates of epithelial (E) and mesenchymal (M) breast cancer cells. Real-time qPCR analysis and protein expression of **C-D**. lactate transporters: MCT1 and MCT4; **E-F.** lactate dehydrogenases: LDHA and LDHB in epithelial (E) and mesenchymal (M) breast cancer cells cultured in 3-dimensional Matrigel with physiological modified medium for 7 days. β-actin was used as a loading control. **G.** NMR profile of lactate doublet, 1.31 ppm, 1.32ppm. pyruvate profile: singlet, 2.37 ppm. **H-I.** Intracellular lactate and intracellular pyruvate of epithelial (E) and mesenchymal (M) breast cancer cells cultured in 3-dimensional Matrigel for 7 days using NMR analysis. The bars represent the mean ± S.E.M. of three independent experiments; (n>4) *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (ordinary one-way ANOVA).

Lactate As A Potential Nutrient In Epithelial Cells

Both epithelial cell lines also exported lactate under MPM culture conditions, albeit at a significantly lower rate than the mesenchymal cells **(Figure 3.4. B)**. In contrast to the mesenchymal cell lines, both epithelial cell lines expressed the lactate and pyruvate transporter, MCT1, at relatively high levels, but expressed the lactate exporter, MCT-4, at very low levels **(Figure 3.4. C-D).** Additionally, NMR analysis indicated higher intracellular pools of pyruvate and lactate in both epithelial cell lines **(Figure 3.4. G-I).** Thus, the two epithelial cell lines maintain an accessible pool of pyruvate for mitochondrial respiration and an accessible pool of lactate that could be converted to pyruvate if needed. As stated above, although the LDHB mRNA levels are low in both epithelial cell lines, LDHA and LDHB protein levels were readily detectable by immunoblot **(Figure 3.4. E-F)**, indicating some capacity for the interconversion of pyruvate and lactate.

The findings above indicated that lactate, as provided through MCT1, can potentially be used as a fuel by the two epithelial cell breast cancer lines. We therefore examined whether MCT1 function promoted cell growth in epithelial cell lines that display robust MCT1 expression **(Figure 3.4. C-D)**. Treatment of cells with the potent MCT1 inhibitor SR13800, slowed the growth of both epithelial cell lines, but had no effect on the mesenchymal cells, in the presence of physiological levels of glucose, lactate, glutamine and pyruvate **(Figure 3.5. A-B)**. In the presence of 0.1 mM, 1 mM or 10 mM lactate but without glucose, glutamine and pyruvate, epithelial cell growth was moderate, but nevertheless was positively correlated with increasing extracellular lactate, and this effect of lactate on cell proliferation was blocked by the MCT1 inhibitor **(Figure 3.5. C-D).** Increasing extracellular lactate had no effect on mesenchymal cells **(Supplemental figure 3.2.).** These data show a differential ability of epithelial breast cancer cells to import lactate for growth.

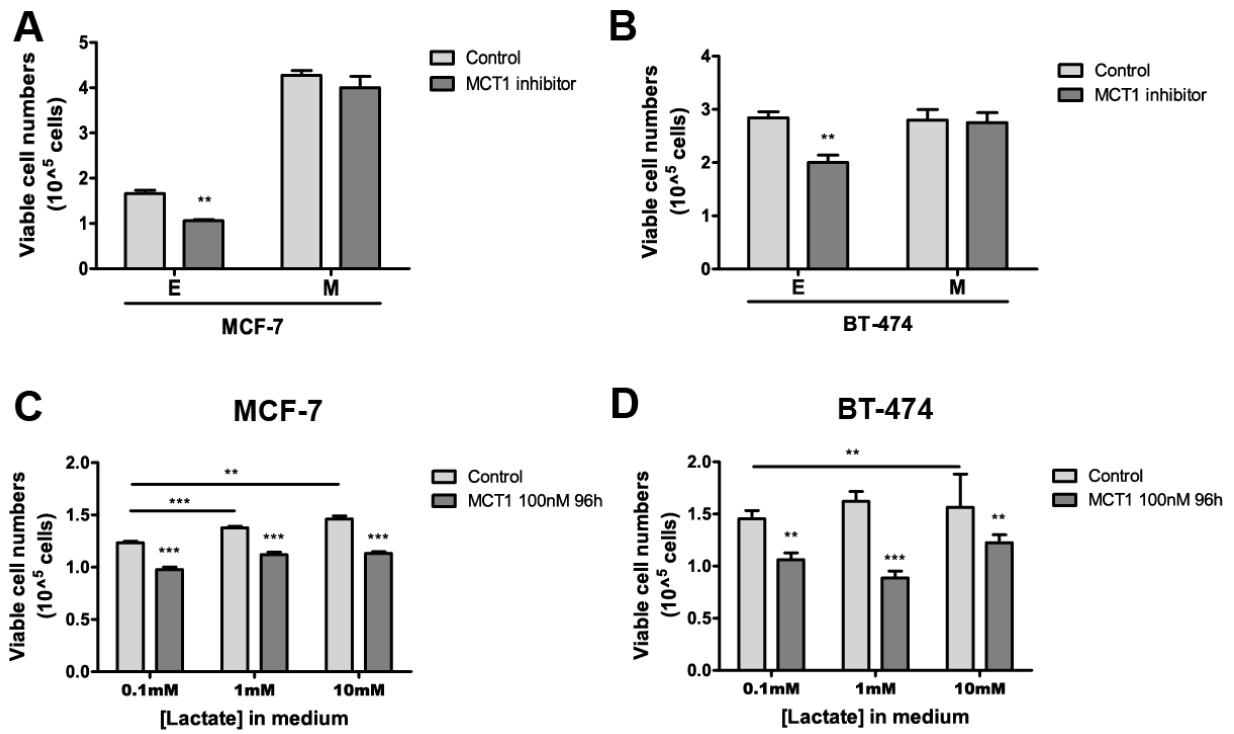


Figure 3.5. MCT1 inhibitor treatment reduced the cell proliferation of epithelial MCF-7 and BT-474 breast cancer cells in normal and nutrient-limited conditions.

Viable cell counts under the following conditions: **A-B.** Epithelial (E) and mesenchymal (M) cells were cultured in 3-dimensional Matrigel with 100 mM MCT1 inhibitor SR 13800 for 4 days. **C-D.** Epithelial MCF-7 and BT-474 cell lines were cultured in 3-dimensional Matrigel with physiological modified medium containing only lactate at 0.1 mM, 1 mM and 10 mM concentrations for 4 days and treated with or without MCT1 inhibitor SR 13800. Results are presented as mean ± S.E.M. of three independent experiments; (n=6) *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (ordinary one-way ANOVA).

GATA3 Expression Promotes MCT1 Expression

The UCSC Genome Browser shows an H3K27Ac mark coinciding with a DNase-hypersensitive region about 5 kb upstream of the human MCT1 transcription start site. Analysis of this region revealed that it harbors a GATA3 binding site **(Figure 3.6. A).** The transcription factor, GATA binding protein 3 (GATA3), is essential for proper mammary development and has strong association with the expression of the estrogen receptor (ERα) in breast cancer[63,64]. We observed that GATA3 knockdown significantly decreased MCT1 and ERα relative expression, but had no effect on MCT4 **(Figure 3.6. B).**

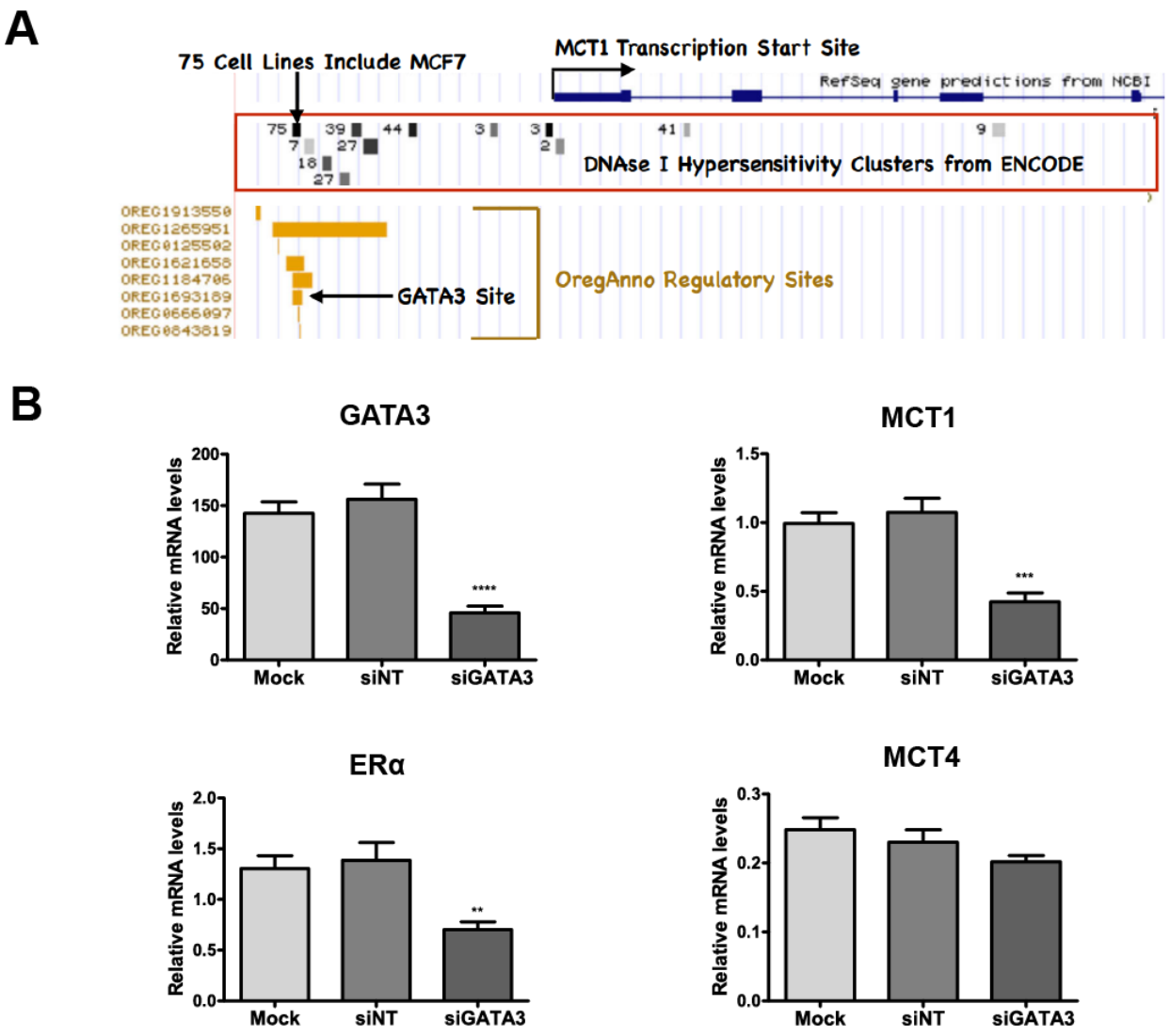


Fig. 6. GATA3 Expression Promotes MCT1 Expression

A. GATA3 binding site in the DNase-hypersensitive region of the human MCT1 gene (UCSC Genome Browser; ORegAnno Regulatory Site Database[65]) **B.** Relative mRNA expression of transcription factor GATA3, lactate transporters MCT1 and MCT4, and estrogen receptor α (ERα) in MCF-7-Mock, MCF-7-siNT and MC-7-siGATA3 for 96 hours in MPM culture. Results are

presented as mean ± S.E.M. of three different experiments (n=6) *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (ordinary one-way ANOVA).

ERα signaling regulates MCT1 expression

The higher expression of MCT1 in ERα-positive epithelial cells raises the possibility that either ERα directly stimulates MCT1 gene transcription, or that ERα/luminal-related transcription factors (e.g., GATA3, FOXA1[66,67]) stimulate MCT1 expression. Indeed, treatment of both epithelial MCF7 and BT474 cell lines with 10 nM E2 or 1 □M Tamoxifen in MPM containing some estrogenic activity (phenol-red, 10% FBS) showed that Tamoxifen significantly suppressed the expression of MCT1 and progesterone receptor (positive control). MCT4 expression was unchanged by either E2 or Tamoxifen, demonstrating a selective action on MCT1 expression **(Figure 3.7. A).** This raised the possibility that Tamoxifen may suppress breast cancer cell proliferation, in part, by lowering expression of MCT1, and that the combination adjuvant therapy with both Tamoxifen and an MCT1 inhibitor may impose a more complete inhibition of growth and viability in ERα-positive breast cancers. Co-treatment of epithelial MCF-7 and BT-474 cells with MCT1 inhibitor SR13800 and Tamoxifen inhibited proliferation to a significantly greater extent than with either drug by itself **(Figure 3.7. B)** but not mesenchymal breast cancer cells **(Supplemental figure 3.3. C).**

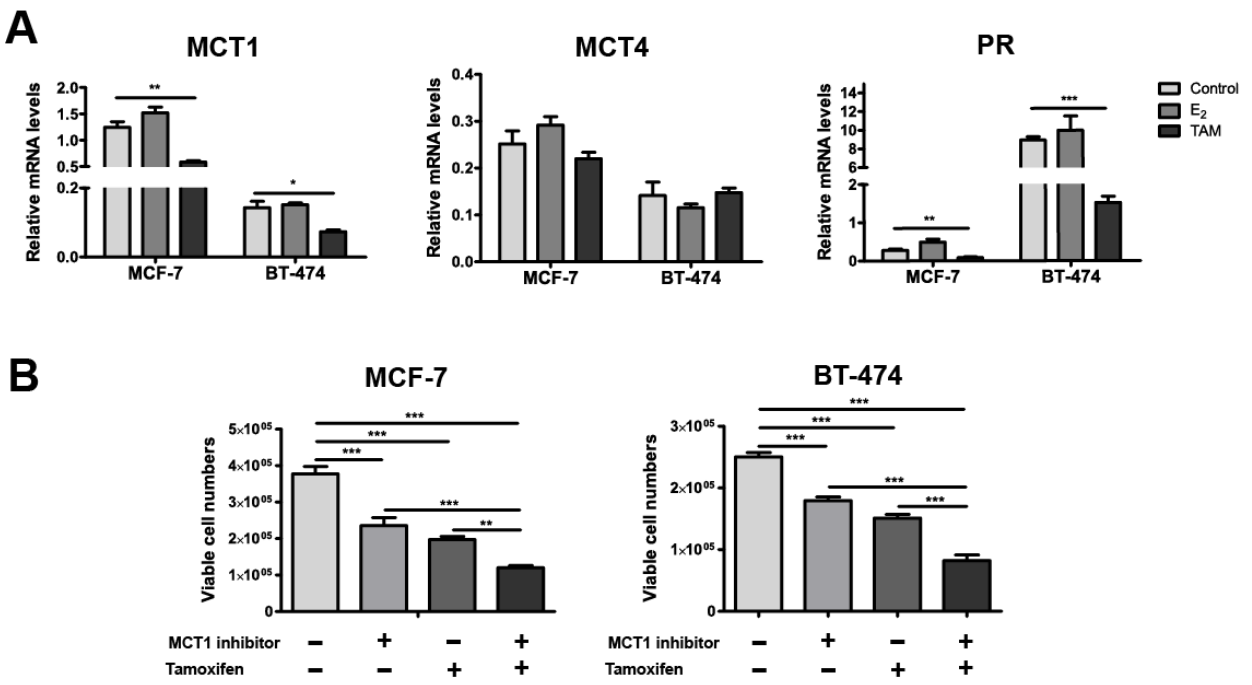


Figure 3.7. ERα signaling regulates MCT1 expression

A. Relative mRNA expression of lactate transporters: MCT1 and MCT4; and progesterone receptor (PR) of epithelial (E) and mesenchymal (M) breast cancer cells in 3-dimensional Matrigel culture and treated with 10 nM β-estradiol (E_2) or 1 μM Tamoxifen (TAM) for 4 days. **B.** Viable cell counts of epithelial MCF-7 and BT-474 breast cancer cells in 3-dimensional Matrigel culture and treated with 1 μM Tamoxifen (TAM) +/- 100 mM MCT1 inhibitor SR 13800 for 4 days. Results are presented as mean ± S.E.M. of three different experiments (n=6) *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (ordinary one-way ANOVA).

IV. Discussion

The behavior and progression of breast cancer is strongly influenced by the tumor microenvironment, which includes the extracellular matrix and the levels of extracellular nutrients. The matrix that surrounds cells in a 3-dimensional field impacts the configuration of the cell, regulates the concentrations of nutrients, exported metabolites and H+ ions, allows cells to generate motile and stress forces, and modulates intracellular signaling pathways[14,68,69].

Extracellular fuels, specifically glucose, lactate, glutamine and pyruvate, contribute to the levels of intracellular metabolites, many of which impose allosteric regulation on metabolic pathways [28,70,71]and enable anabolic pathways required for cell proliferation. For example, Elia et al recently demonstrated that 3D culture of Ras-transformed MCF10A mammary epithelial cells in soft agar induced a shift towards proline metabolism, and that cells in 3D culture also imported proline, whereas cell in 2D culture exported proline[72].

Therefore, we revisited and extended our previous studies on EMT and metabolic reprogramming[47] by adapting a 3-dimensional culture method in Matrigel, and using a modified culture medium with 4 major nutrients at approximately physiological concentrations ("Matrigel/physiological medium" or "MPM"). In MPM culture, the two epithelial and two derivative mesenchymal cell lines displayed marked morphological differences and motility/invasiveness that is difficult to perceive in a constrained 'flat' plain of the traditional 2-dimensional plastic culture. In addition to increased invasiveness within the Matrigel, the two post-EMT mesenchymal cell lines displayed a triple-negative phenotype with increased proliferation, and expressed EMT-related and stem cell-related markers under MPM culture conditions.

Previous studies on metabolic reprogramming in the context of metastatic transformation or EMT suggest that this process facilitates the metabolic shift toward glycolysis. For example, the EMT-related transcription factor, SNAIL, mediates the suppression of mitochondrial respiration through the Wnt/Snail signaling pathway, and enhances glycolysis by silencing the gluconeogenic enzyme fructose-1,6-bisphosphatase 1 (FBP1) in breast cancer[58,73]. In a previous study, we reported that the two mesenchymal cell lines, MCF-7_{EMT} and BT-474_{EMT} underwent an apparent glycolytic shift, as evidenced by enzyme and transporter expression, glucose uptake, lactate production and relative sensitivities to several metabolic inhibitors[47].

In the present study, we observed that EMT-induced cancer cells had increased glucose uptake and lactate production when cultured in 3-dimensional MPM. We also examined in real time the metabolic changes induced by EMT using the Seahorse extracellular flux technology in all four cell lines and in physiological modified medium. The significantly lower levels of OCR in the EMT-induced mesenchymal breast cancer cells were accompanied by an increase in ECAR levels when compared to the parental epithelial cell lines, confirming a glycolytic shift in the mesenchymal cell lines.

Acidification of the extracellular fluid (i.e., an enhanced ECAR) is generated primarily by the co-export of lactate anion and a proton. Lactate is generated from pyruvate, the end product of glycolysis, by lactate dehydrogenase isoform A (LDHA) -enriched tetramers. This reaction also oxidizes NADH to NAD+ that is essential for the continuation of a high glycolytic flux. Tetramers enriched in LDHB catalyze the reverse reaction of lactate to pyruvate. We observed increased protein levels of both LDHA and LDHB in the two mesenchymal cell lines. LDHA overexpression is a hallmark of many types of cancer, the majority of which are highly glycolytic, and elevated levels of LDHA connotes poor prognosis in pancreatic, squamous head and neck, gastric and breast cancer[55,74–76]. In contrast, the association of LDHB with breast cancer is more complex. LDHB, which converts lactate to pyruvate, was shown to be silenced by promoter methylation in breast cancer cells under hypoxia to enhance glycolysis[77]. However, one study associated high levels of LDHB with a more glycolytic and basal-like phenotype among several breast cancer cells [77]. Moreover, another study showed that LDHB was highly expressed in triple-negative breast cancer as compared with other subtypes of breast cancer and patients with elevated LDHB experienced poor clinical outcome [78]. Further studies are needed to examine whether the LDH isoforms are modified in cancer cells in a manner that confers either different enzymatic actions with respect to

pyruvate and lactate, or entirely new actions that are unrelated to the interconversion of these two metabolites.

The co-export of lactate and a proton is accomplished by specific isoforms of the monocarboxylate transporter (MCT) family[79]. While the release of lactate occurs through the low-affinity lactate transporter MCT4 (Km≈28 mmol/L), the import of lactate occurs through the high-affinity MCT1 transporter (Km≈1– 3.5 mmol/L). In normal physiology, MCT4 is expressed in highly glycolytic tissues like type II skeletal muscle fibers and astrocytes and MCT1 is highly expressed highly oxidative tissues such as cardiac muscle, type I skeletal muscle fibers, and neurons [26,79]. In our study, we observed that both EMT-induced mesenchymal cell lines displayed increased levels of the MCT4 transporter, and diminished levels of MCT1. The relative expression of MCT isoforms in relation to different types of breast cancer is not straight forward. Doyen et al reported that the expression of MCT4 was higher in TNBC compared to normal breast tissues [80] and supports the correlation of elevated expression of MCT4 in the triple-negative mesenchymal cancer cells. In contrast, other studies have demonstrated a strong association of high expression of MCT1 with triple-negative breast cancer compared to other breast cancer types [81,82]. However, one study reported that only 26% of their 31 TNBC samples showed high expression of MCT1 [78]. Interestingly, a recent report showed that MCT1 and MCT4 were co-expressed in some breast cancer tumors and cell lines [83]. One possible explanation for these discrepancies is the plasticity of tumor cells to adapt to different tumor microenvironments. For example, the relative degree of vascularization and oxygen saturation in individual samples are typically not reported in studies of differential gene expression among different breast cancer types.

We observed lower steady state levels of intracellular pyruvate and lactate in the mesenchymal cells, which, coupled to the findings of elevated glucose uptake, increased LDH and

MCT4 expression and increased lactate secretion, indicates that glycolytic cells are extremely efficient at lactate production and removal. Interestingly, lactate secreted by more glycolytic tumor cells, has been identified as a major energy fuel in tumors. Lactate is taken up by oxygenated tumor cells which use it to fuel oxidative metabolism[27,84]. Thus, oxidative tumor cells spare glucose which may, in turn, reach glycolytic tumor cells. This "lactate shuttle" between tumor cells has been described in different types of cancer including breast cancer [28].

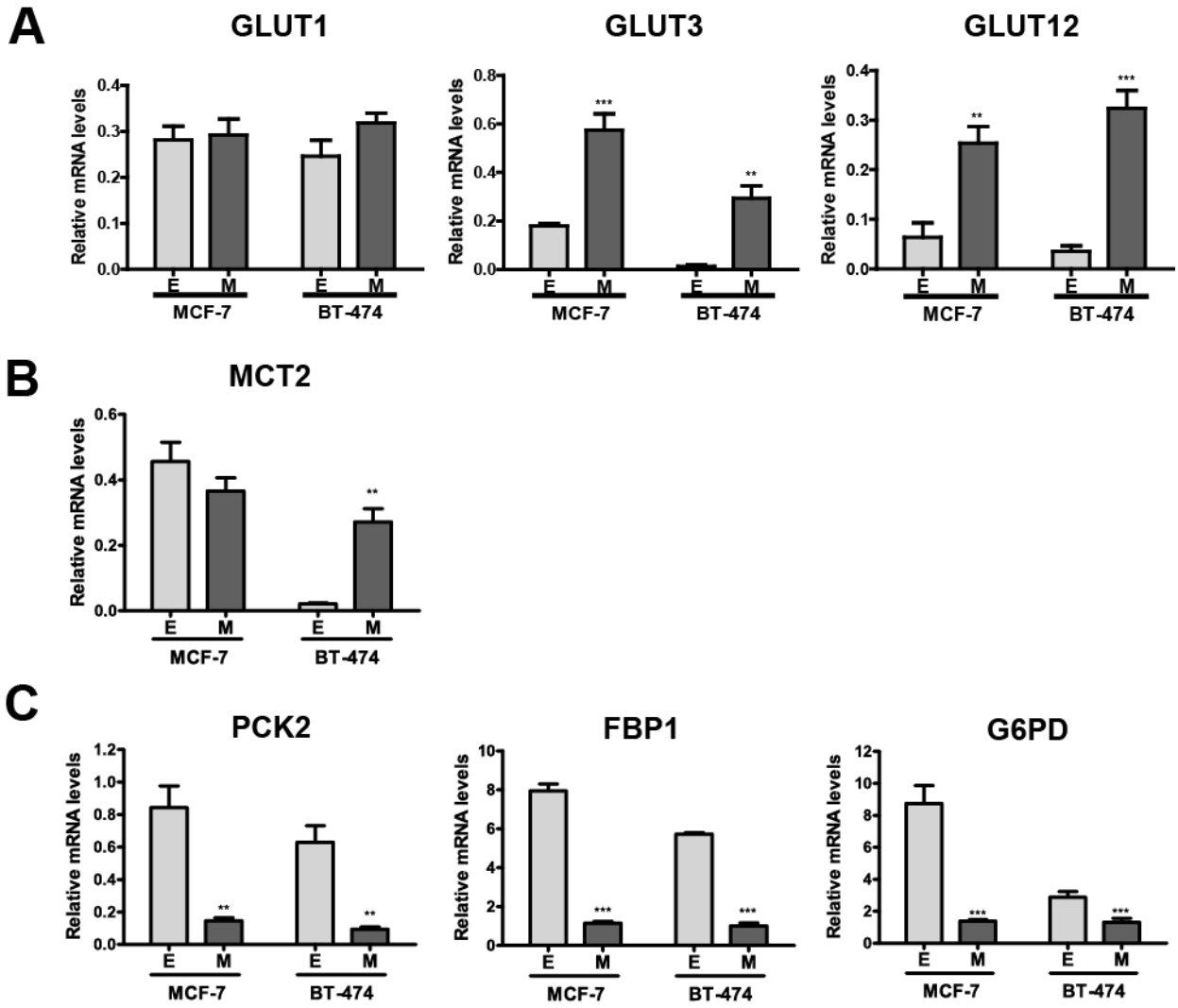
In contrast to the two EMT-induced mesenchymal cell lines, the two parental epithelial cell lines displayed a higher OCR but a lower ECAR. Further, the epithelial cells contained significantly more intracellular pyruvate and lactate. The epithelial cells expressed robust levels of MCT1 protein but very low levels of MCT4, and readily detectable levels of both LDH isoforms. These findings indicate that epithelial cells import lactate, convert lactate to pyruvate and maintain a reservoir of both metabolites to be used to maintain mitochondrial oxidative metabolism. Our previous study[47] and the findings shown in Supplemental Fig 1 indicate that epithelial cells express enzymes that can redirect carbons in pyruvate through reverse glycolysis and into anabolic and antioxidant pathways. Thus, MCT1-mediated lactate uptake also likely subserves the ability of epithelial cells to double their mass for proliferation.

Lactate also functions independently of providing a carbon source, in that elevated levels of intracellular lactate may also regulate intracellular signaling pathways in epithelial cells[85]. For example, in oxidative cancer cells, lactate has been linked to activation of a "pseudo-hypoxic" Hif-associated signaling, leading to activation of the c-myc signaling pathway, ultimately leading to an increase in glutamine uptake and metabolism[39]. Given the complexity of lactate actions, the MCT1 inhibitor likely decreases the viable cell number for the two epithelial cell lines through perturbation of multiple pathways. Further studies are needed to examine whether and how the

higher level of intracellular lactate in the two epithelial cells impacts signaling pathways that regulate other metabolic pathways, as this information may allow for higher precision in the inhibition of lactate intake and function to impair cancer cell metabolism (and lead to cell death) without allowing metabolic adjustments to override the impairments.

Consistent with the fact that MCT1 mRNA and protein expression is significantly higher in ERα-positive epithelial cells, Tamoxifen suppresses MCT1 expression. The combination of Tamoxifen and an MCT1 inhibitor had an additive effect on the decline in cell number in the two epithelial cell lines. Also consistent with expression and function of MCT1 in epithelial Luminal A breast cancer cells, GATA3 also selectively stimulates MCT1 expression. Further studies are needed to better exploit this interaction of luminal transcription factors, ERα and GATA3, with MCT1 and lactate metabolism for therapeutic targeting of Luminal A breast cancer cells.

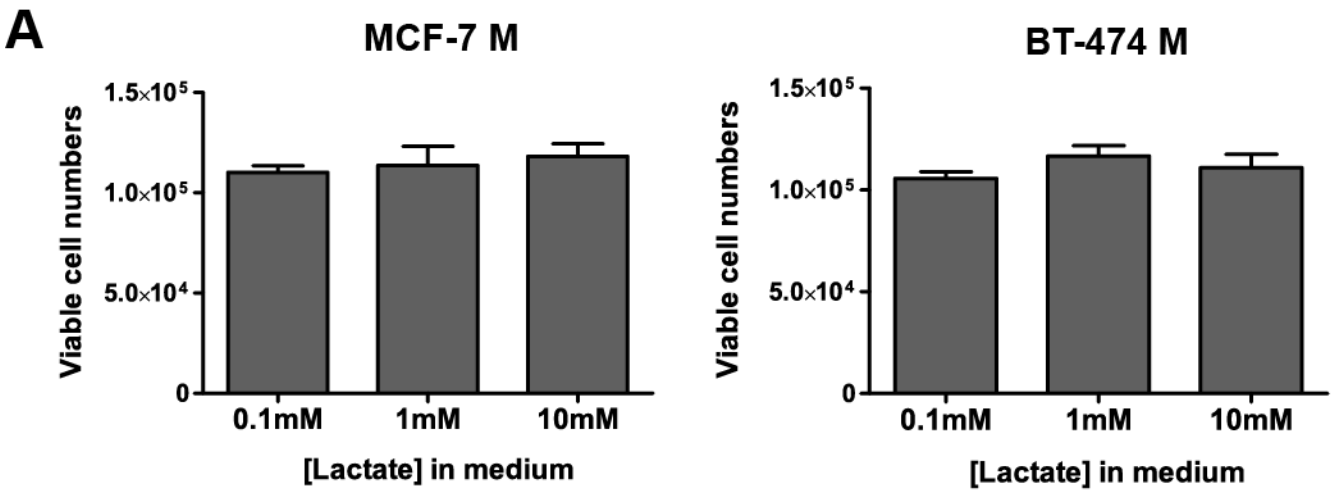
V. Supplemental figures



Supplemental figure 3.1. EMT-induced BT-474M and MCF-7M mesenchymal breast cancer cells showed upregulation of glucose transporters and downregulation gluconeogenic enzymes.

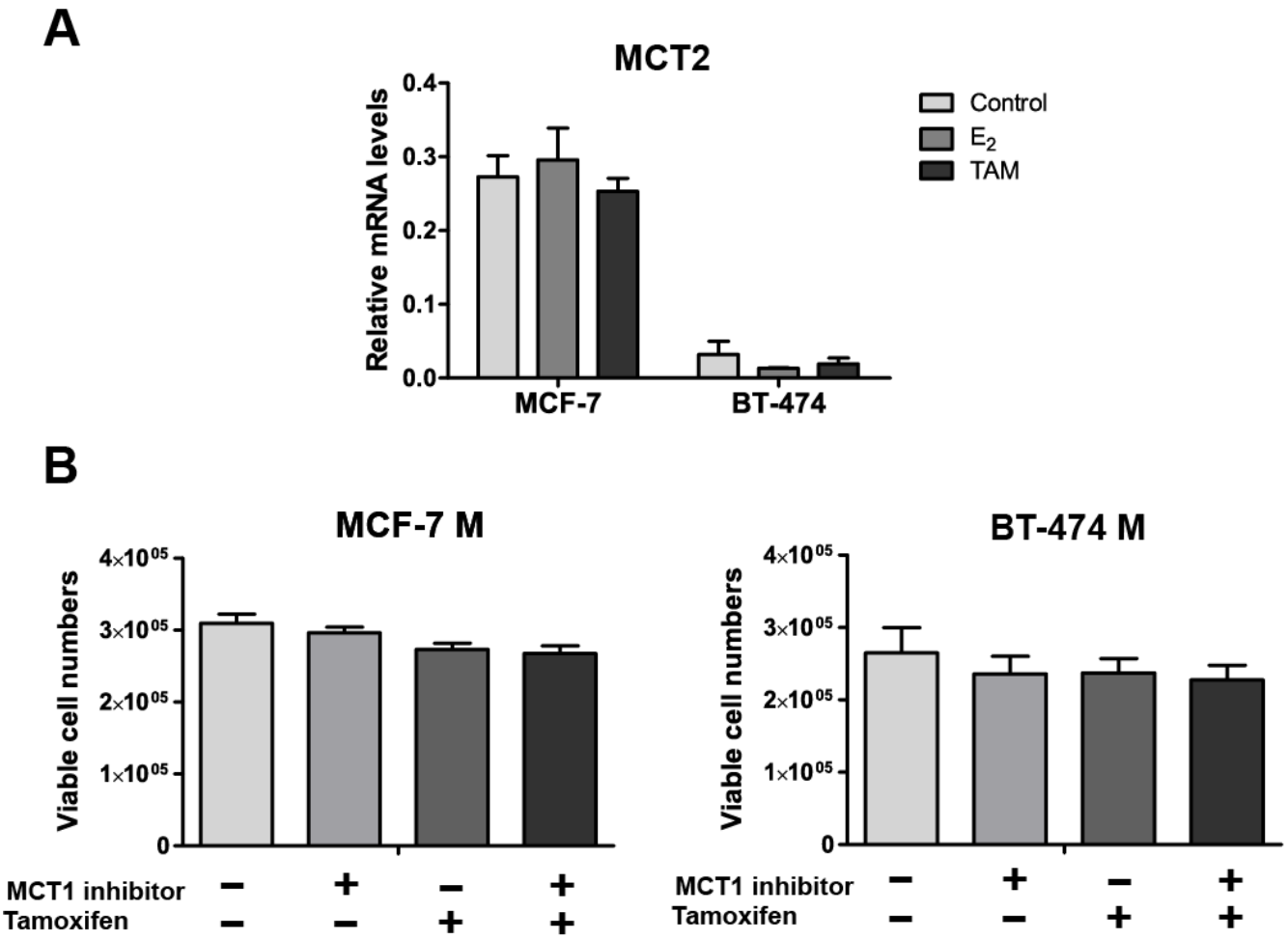
Real-time qPCR analysis of **A**. glucose transporters; GLUT1, GLUT3 and GLUT12. **B.** lactate transporter; MCT2. **C.** gluconeogenic enzymes; PCK2, FBP1 and G6PD. Results are presented as

mean ± S.E.M. of three independent experiments (n>6) *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (ordinary one-way ANOVA).



Supplemental figure 3.2. Cell proliferation of the EMT-induced mesenchymal MCF-7M and BT-474M breast cancer cells culture in 3-dimensional Matrigel and nutrient-limited medium.

A. Mesenchymal MCF-7 M and BT-474 M cell lines were cultured in 3-dimensional Matrigel with physiological modified medium containing only lactate at 0.1 mM, 1 mM and 10 mM concentrations for 4 days. Results are presented as mean ± S.E.M. of three different experiments (n=6) *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (ordinary one-way ANOVA).



Supplemental figure 3.3. Tamoxifen and MCT1 inhibitor treatment does not affect the cell proliferation of the EMT-induced mesenchymal MCF-7M and BT-474M breast cancer cells. **A.** Relative mRNA expression of lactate transporters; MCT2 of epithelial (E) and mesenchymal (M) breast cancer cells in 3-dimensional Matrigel culture and treated with 10 nM β-estradiol (E_2) or 1 µM Tamoxifen (TAM) for 4 days. **B.** Viable cell counts of mesenchymal MCF-7M and BT-474M breast cancer cells in 3-dimensional Matrigel culture and treated with 1 µM Tamoxifen (TAM) +/- 100 mM MCT1 inhibitor SR 13800 for 4 days. Results are presented as mean ± S.E.M. of three different experiments (n=6) *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ (ordinary one-way ANOVA).

CHAPTER 4

GPR81 REGULATES LACTATE METABOLISM IN EPITHELIAL BREAST CANCER CELLS

I. Abstract

G-protein-coupled receptor 81 (GPR81) functions as a receptor for lactate and plays an important role in cancer progression. However, the expression of GPR81 and its role in the context of metastatic cancer have not been previously reported. Epithelial-mesenchymal transition (EMT) promotes metastasis by inducing invasive properties in epithelial tumors. Here, we have studied the expression of GPR81 in two epithelial breast cancer cell lines (MCF-7 and BT-474) and their respective EMT-induced mesenchymal progeny (MCF-7 M and BT-474 M). GPR81 was highly expressed in epithelial breast cancer cell lines and hormone-positive breast cancer tumors but was suppressed in post-EMT mesenchymal breast cancer cells or triple negative breast cancer (TNBC) tumors. To determine the role of GPR81 in epithelial breast cancer cells, we silenced the lactate receptor. GPR81 knockdown resulted in impaired breast cancer cell growth and led to apoptosis. We found that GPR81 regulated the expression of the lactate importer, monocarboxylate transporter 1 (MCT1), and the lactate uptake in epithelial breast cancer cells. GPR81 expression was crucial for breast cancer cell proliferation and survival under nutrient-limited conditions. Our study indicates that GPR81 might be a potential prognostic marker and therapeutic target in hormone positive epithelial breast cancer.

II. Introduction

Breast cancer is a complex and heterogeneous disease, which includes a range of histologic subtypes associated with different clinical behavior and patient's outcomes. Invasive breast cancer (IBC) is the most predominant form of cancer among women worldwide and the second cause of cancer-related deaths in women in the United States [5,51]. The most common type of IBC is luminal A, characterized by an adhesive epithelial phenotype and defined by the overexpression of the estrogen receptor α (ERα) and progesterone receptor (PR). Although Luminal A IBC is a relatively non-aggressive type and has a good response to endocrine therapy, failure of early detection is associated with metastatic disease, which in turn is responsible for the majority of deaths in breast cancer patients.

Metastatic dissemination of IBC starts when cancer cells penetrate the basement membrane into nearby breast tissue. Invasive breast cancer cells travel to other parts of the body through the lymphatic system or the circulation, ultimately leading to colonization of distant organs[52]. Epithelial-mesenchymal transition (EMT) has been proposed as an early step in the metastatic process[8,52]. EMT modifies the adhesion molecules expressed by the cell allowing detachment from the main tumor mass, and increases motility and invasiveness of previously indolent tumor cells [8]. The process of EMT appears to be induced by exposure of cancer cells to an altered microenvironment (e.g., hypoxia, increased cytokines), sometimes in conjunction with additional somatic mutations [10 12].

We previously reported that prolonged mammosphere culture induced EMT in two distinct epithelial breast cancer cell lines, MCF-7 and BT-474 cells, generated stable populations of mesenchymal cancer cells, MCF-7M and BT-474M [47,48]. More recently, we showed that EMT promoted a more glycolytic phenotype compared to the parental epithelial breast cancer cells that

primarily use oxidative phosphorylation to produce ATP (Chapter 3). The altered metabolic phenotype induced by EMT involved enhanced aerobic glycolysis along with higher rates of lactate production.

Several studies have highlighted lactate as a "signaling molecule" that plays a role in cancer cell migration, angiogenesis, immune escape and metastasis[25,37]. Elevated levels of lactate within the tumor microenvironment have been associated with metastasis and poor prognosis of cancer patients [27,31,34,86–88] Additionally, lactate produced by highly glycolytic cancer cells can be utilized by neighboring oxidative cancer cells for ATP production, a phenomenon known as "metabolic symbiosis"[27]. Specifically, one study showed that human breast cancer tumors exhibited lactate accumulation ranging from 0.5 mM to 8 mM, which under some circumstances is used as an alternative energy source by oxidative breast cancer tumor cells[28].

Intracellular and extracellular lactate levels are determined, in part, by different isoforms of the lactate/H^+ symporter, termed monocarboxylate transporters (MCTs). These isoforms have different affinities for lactate and pyruvate, which influence the direction of lactate flux[79]. MCT1 has a high affinity for lactate and is primarily responsible for lactate influx. Clinical studies for MCT1 inhibition are being conducted as a potential anti-cancer treatment option for advanced solid tumors in patients with lymphoma, prostate and gastric[89]. In contrast, MCT4 has lower affinity for lactate and is primarily involved in lactate export, which contributes to the acidification and lactate accumulation in the tumor microenvironment [26,90]. Additional important players in lactate shuttling between cancer cells are the lactate dehydrogenases A and B (LDHA and LDHB) that catalyze the reversible conversion of pyruvate to lactate.

Previous studies have identified the cell-surface G-protein-coupled receptor, GPR81, as an endogenous lactate receptor. The GPR81 family of receptors consists of three highly homologous

members, GPR109a, GPR109b and GPR81, which are regulated by the specific agonists, 3-hydroxybutyrate, 3-hydroxyoctanoate, and lactate, respectively [41]. GPR81 has been mainly studied in adipocytes, in which the receptor is coupled to Gi/q. GPR81 activation by lactate decreases cAMP production, which ultimately reduces lipolysis [42]. Recently GPR81 has been shown to be upregulated in most cancers (pancreas, colon, liver, breast, cervix) and its expression levels associated with tumor growth and metastasis [43–46,91]. In this current study, we examined GPR81 expression in the context of metastatic transformation or EMT in breast cancer. We provide evidence that GPR81 promotes lactate transport in hormone-positive breast cancer cells and its expression is crucial for their cell proliferation and survival.

III. Results

Endogenous Lactate Receptor GPR81 Is Highly Expressed In Epithelial Breast Cancer Cells In 3-Dimensional Culture

As previously described, epithelial (E) MCF-7 and BT-474 cell lines and the corresponding post-EMT mesenchymal (M) MCF-7M and BT-474M were cultured in a more physiologically relevant system using 3-dimensional Matrigel and physiological modified medium (referred as MPM culture). Under these conditions epithelial breast cancer cells grew as tightly adherent spheroids for 1 week **(Figure 4.1. A; "E" boxes).** The two mesenchymal cell lines also formed tight spheroids initially, but after 2 days began invading the Matrigel **(Figure 4.1. A; "M" boxes)**. After 1 week of MPM culture, cells were examined for the expression of the endogenous lactate receptor GPR81. Epithelial spheroids expressed relatively high levels of GPR81, whereas mesenchymal cells showed no or very low expression **(Figure 4.1. B).**

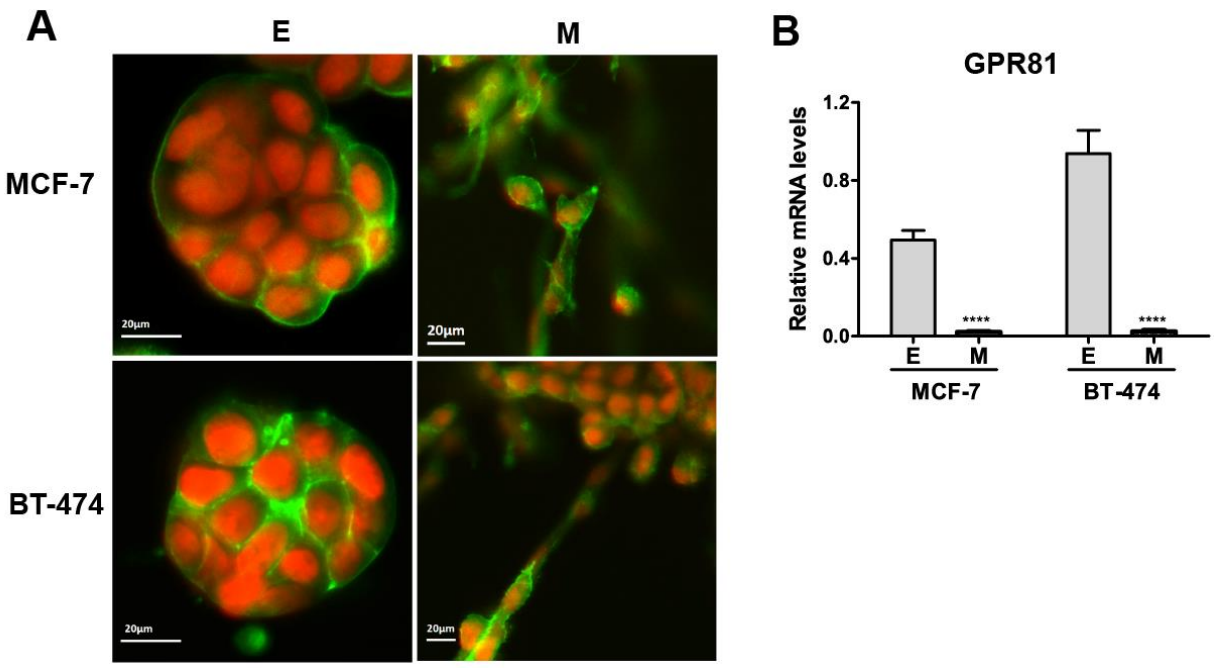


Figure 4.1. GPR81 expression of epithelial MCF-7 and BT-474 cells and mesenchymal MCF-7M and BT-474M in 3-dimensional Matrigel culture.

A. Fluorescence microscopy images of 3-dimensional structures of proliferating epithelial (E) and mesenchymal (M) cells in 3-dimensional Matrigel with physiological modified medium for 7 days. Green, Phalloidin staining of F-actin; Red/orange, nuclei counterstained with SYTOX^{TM} orange nucleic acid (red). **B.** Real-time qPCR analysis of GPR81 in epithelial (E) and mesenchymal (M) cells. Results are presented as mean ± S.E.M. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ (ordinary one-way ANOVA).

GPR81 Is Highly Expressed In Human Hormone-Receptor-Positive Breast Cancer Tissues

To determine whether GPR81 was present in human breast cancer tumors, we analyzed mRNA gene expression profiles from 74 human breast cancer samples and 12 non-tumorigenic (NT) breast tissues **(Supplemental figure 4.1. B).** We grouped these samples in non-tumorigenic (NT), estrogen receptor positive (ER^+), amplification of the human epidermal receptor growth

factor 2 ($HER2^+$) and triple negative breast cancer (TNBC) according to their clinical information provided by the supplier. Higher levels of GPR81 mRNA were noted in hormone-positive breast cancer samples (ER^+ and $HER2^+$) and significantly lower levels were found in the NT and TNBC samples **(Figure 4.2. A).** We also analyzed the relative gene expression of GPR81 by the stage of human breast cancer. Breast cancer stage II-III had significantly higher GPR81 expression than stage IV **(Figure 4.2. B).** Additionally, the Kaplan-Meir survival plot showed that high expression levels GPR81 was strongly associated with better prognosis and overall survival in breast cancer patients **(Figure 4.2. C)** (p-value = 0.0011) (n = 626)[92]. We also tested GPR81 expression in other breast cancer cell lines. Hormone-receptor positive HC1500 cells had higher expression of the lactate receptor compared to the TNBC MDA-MB231 cells **(Supplemental figure 4.1. A)**. These results demonstrate that GPR81 is mainly expressed in hormone positive and less aggressive types of human breast cancer.

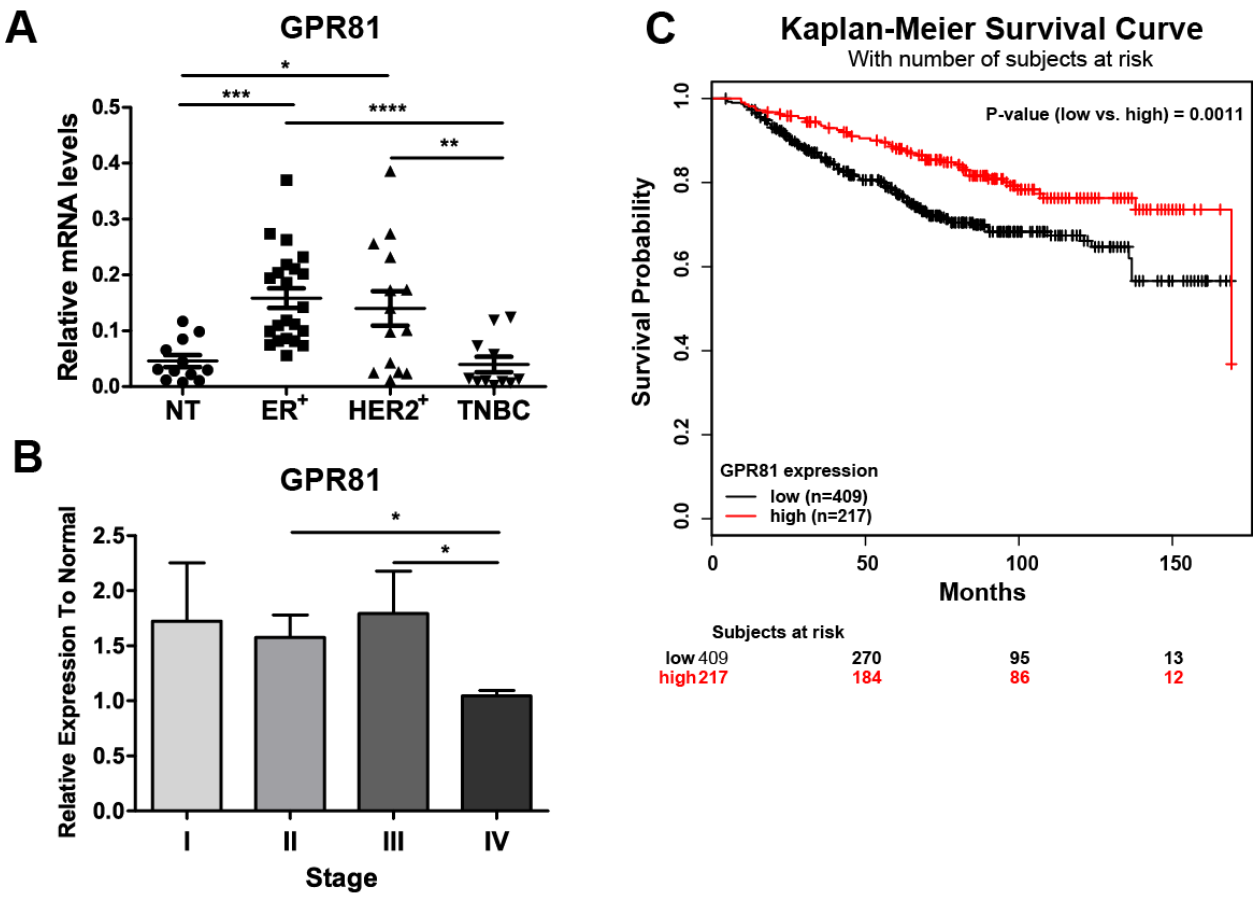


Figure 4.2. GPR81 is highly expressed in human hormone-receptor-positive breast cancer tissues.

A. Relative mRNA expression of GPR81 in NT (n=12) ER^+ (n=23), $HER2^+$ (n=14) and TNBC (n=11) breast cancer tissue. **B.** Relative mRNA expression of GPR81 in stage I (n=21), II (n=32), III (n=25) and IV (n=4) of breast cancer. **C.** Kaplan-Meier plot of estimated overall survival in breast cancer patients (n=626) with low expression of GPR81, below the median (black line) or high expression of GPR81, above the median (red line)[92]. Log-rank test p-value = 0.0011. Results are presented as mean ± S.E.M. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (two-way ANOVA).

GPR81 regulates expression of the gene involved in lactate import in MCF-7 epithelial breast cancer cell line

A previous study reported that GPR81 regulates the expression of lactate transport related genes MCT1 and MCT4 in pancreatic cancer cells through as yet unknown mechanisms[44]. To determine the role of the endogenous lactate receptor GPR81 in the lactate metabolism of ERα-positive breast cancer cells, we silenced GPR81 (~60%) in MCF-7 cancer cells using siRNA (siGPR81) up to four days in MPM culture **(Figure 4.3.A).** GPR81 knockdown in MCF-7 cells led to a 2-fold reduction in mRNA and protein expression of the lactate importer, MCT1, but had no significant effect on the lactate exporter, MCT4 **(Figure 4.3. B-C).** In addition, GPR81 did not significantly alter the expression of other lactate metabolism-related genes (LDHA, LDHB, MCT2) **(Supplemental Figure 4.2.).**

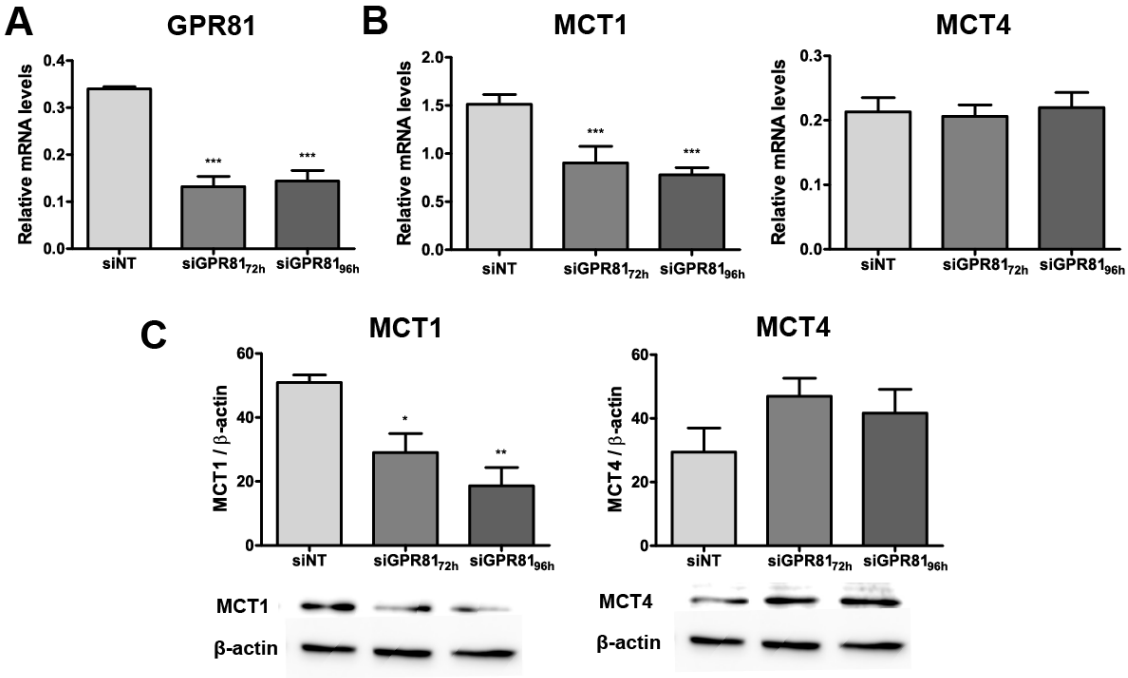


Figure 4.3. GPR81 regulates expression of the gene involved in lactate import in MCF-7 epithelial breast cancer cell line.

A. Real-time qPCR analysis of GPR81, **B.** MCT1 and MCT4 mRNA levels in MCF-7-siNT (control) and MCF-7-siGPR81 cultured in 3-dimensional Matrigel with physiological modified medium at 72 and 96 hours after transfection. **C.** Western blot analysis of cell lysates from MCF-7-siNT and MCF-7-siGPR81 cells used to detect protein expression levels of MCT1 and MCT4. β-actin was used as a loading control. The bars represent the mean ± S.E.M of three independent experiments; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ (ordinary one-way ANOVA).

GPR81 regulates lactate uptake in MCF-7 epithelial breast cancer cell line

We previously reported that the two epithelial breast cancer cell lines had higher steady state levels of intracellular lactate and pyruvate than the corresponding post-EMT mesenchymal cell lines (see Chapter 3). Given that MCT1 is primarily a lactate importer, we hypothesized that the reduction of MCT1 expression may affect the lactate uptake of the epithelial MCF-7 breast cancer cells. To test this hypothesis, we measured the intracellular lactate of non-targeted control MCF-7-siNT and MCF-7-siGPR81 in MPM culture using NMR analysis. MCF-7-siGPR81 cells had significantly lower intracellular lactate compared to MCF-7-siNT cells **(Figure 4.4. A)**, indicating reduced lactate uptake of MCF-7-siGPR81 cells. Additionally, GPR81 knockdown resulted in higher levels of lactate in the culture medium but not control MCF-7-siNT cells **(Figure 4.4. B).** We did not detect a significant change in glucose consumption **(Figure 4.4. C).** Taken together, these data suggest a role for GPR81 in the regulation of the lactate uptake of the epithelial MCF-7 breast cancer cells.

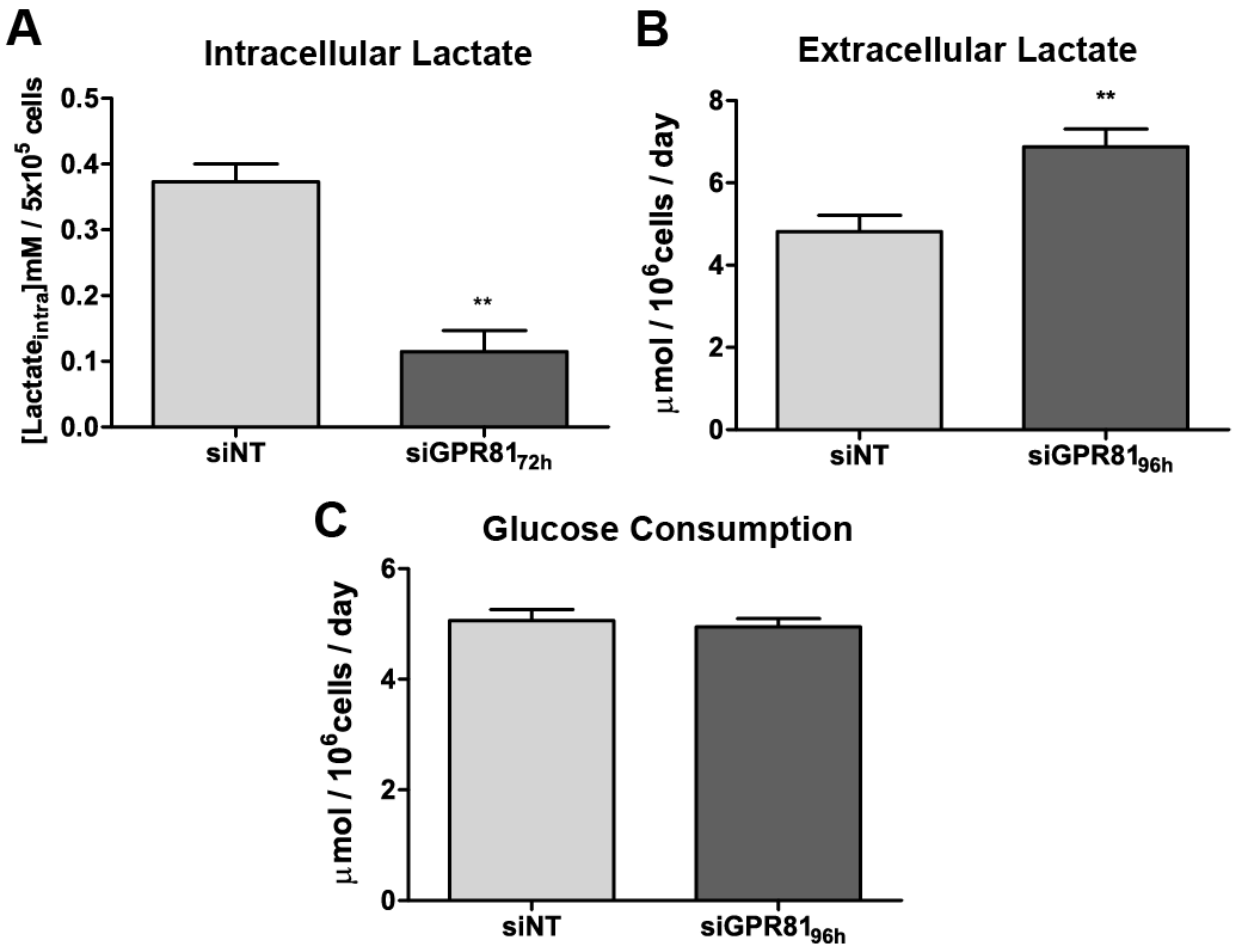


Figure 4.4. GPR81 regulates lactate uptake in MCF-7 epithelial breast cancer cell lines. Intracellular lactate of MCF-7-siNT (control) and MCF-7-siGPR81 measured using NMR analysis after 72 hours of transfection. **B.** Lactate concentration in the medium of MCF-7-siNT 10 nM and MCF-7-siGPR81 10 nM were measured after 96 hours of transfection. **C.** Glucose consumption rate of MCF-7-siNT and MCF-7-siGPR81 were measured after 96 hours of transfection. MCF-7-siNT and MCF-7-siGPR81 cells were cultured and transfected in 3-dimensional Matrigel with physiological modified medium and measurements were taken after 24 hours of culture in fresh medium. The bars represent the mean ± S.E.M of three independent experiments; *$p < 0.05$; **$p < 0.01$; (ordinary one-way ANOVA).

GPR81 is required for cancer cell proliferation and cancer cell survival when lactate is the primary fuel source.

Lactate has been previously suggested as an alternative energy source for aerobic breast cancer cells[27,28,93]. To determine whether GPR81 plays a role in breast cancer cell proliferation, we studied the increase in viable cell numbers and the expression of cell proliferation markers (taken from the OncotypeDx recurrence score assay) in control MCF-7-siNT and MCF-7-siGPR81 cells. Silencing of GPR81 led to about 40% reduction in cell proliferation of MCF-7-siGPR81 **(Figure 4.5.A)** and decreased relative gene expression of proliferation markers; Ki67, Cyclin B1, PCNA and STK15 **(Figure 4.5. B).** Furthermore, the expression of the cell apoptosis marker, cleaved PARP, was significantly increased by 2-fold in MCF-7-siGPR81 cells when compared to control MCF-7-siNT cells **(Figure 4.5. C).**

In order to determine whether GPR81 expression is required for cell growth and survival, we cultured control MCF-siNT and MCF-7-siGPR81 cells in 3-dimensional Matrigel with physiological medium lacking glucose, glutamine and pyruvate and with 0.1 mM, 1 mM or 10 mM lactate as the main available nutrient source. Despite the significant increase in cell number of the MCF-7-siNT cells when cultured in medium containing only 1 mM and 10 mM lactate, GPR81 silencing prevented cell growth under these nutrient-limited conditions. Similarly, we found that treatment with the MCT1 inhibitor also prevented cell growth when in MCF-7 cells were culture in nutrient-limited conditions. Taken together, these data support the physiological role for GPR81 in the regulation of lactate uptake, cell proliferation and survival of the epithelial MCF-7 breast cancer cells under nutrient-limited conditions.

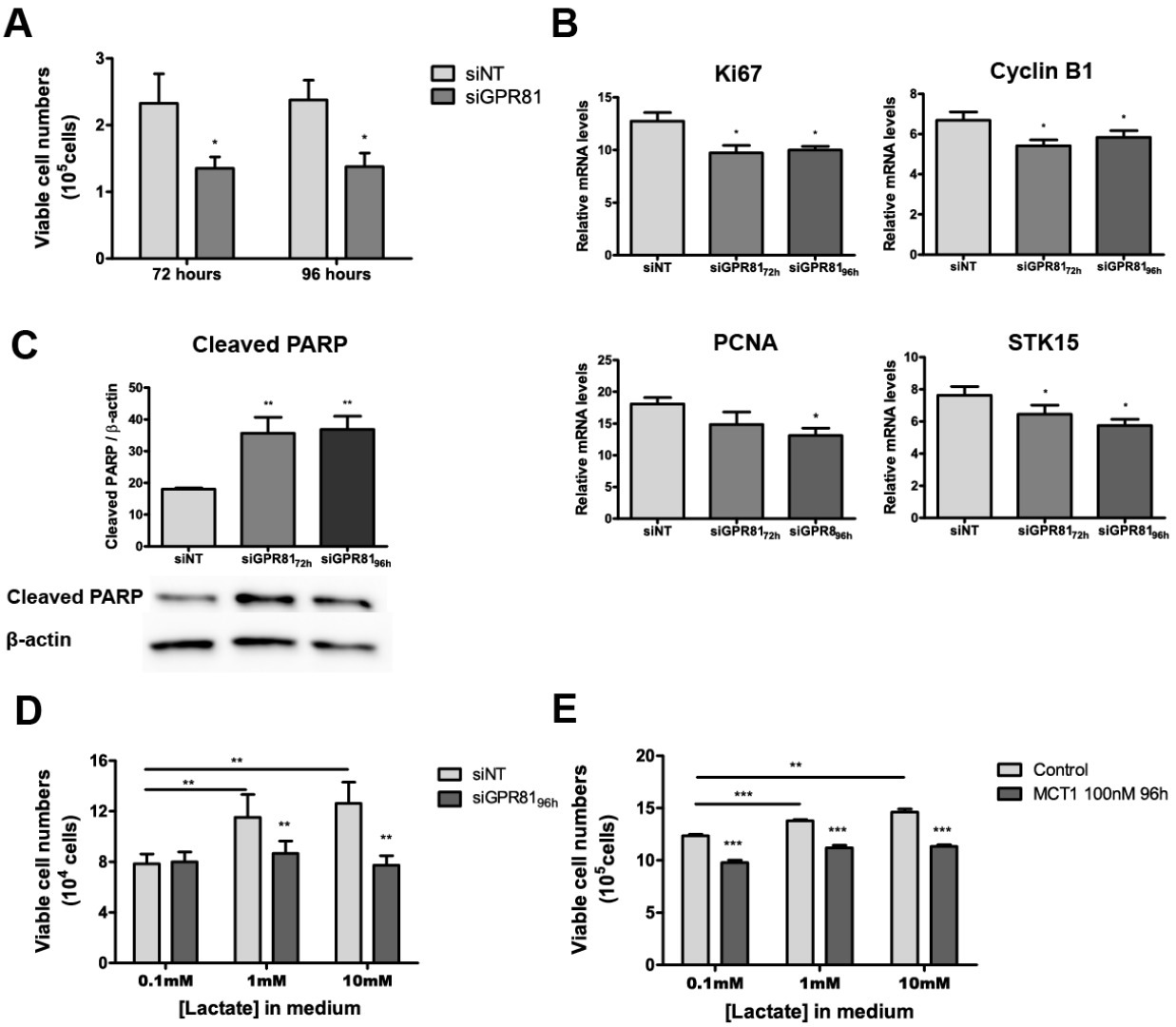


Figure 4.5. GPR81 is required for cancer cell proliferation and cancer cell survival when lactate is the primary fuel source.

A. Viable cell counts of MCF-7-siNT (control) and MCF-7-siGPR81 after 72 and 96 hours of transfection and subtracting the initial cell number. **B.** Real-time qPCR analysis of proliferation markers: Ki67, Cyclin B1, PCNA and STK15. **C.** Western blot analysis of cell lysates from MCF-7-siNT and MCF-7-siGPR81 cells after 72 and 96 hours of transfection used to detect protein expression levels of the apoptotic maker cleaved-PARP. β-actin was used as a loading control. **D**.

MCF-7-siNT and MCF-7-siGPR81 breast cancer cells were cultured in 3-dimensional Matrigel with physiological modified medium containing only lactate at 0.1 mM, 1 mM and 10 mM concentration for 96 hours. **E.** MCF-7 breast cancer cells were cultured in 3-dimensional Matrigel with physiological modified medium containing only lactate at 0.1 mM, 1 mM and 10 mM concentration and treated with or without MCT1 inhibitor SR 13800 for 96 hours. Viable cell counts after treatment. Results are presented as mean ± S.E.M. of three independent experiments; *p < 0.05; **p < 0.01; ***p < 0.001 (ordinary one-way ANOVA).

Additive effect of GPR81 knockdown and Tamoxifen treatment in reducing the cell proliferation and increasing cell apoptosis in epithelial MCF-7 breast cancer cells.

Previously, we showed that 1 µM Tamoxifen significantly reduced the expression of MCT1 in epithelial breast cancer cell lines (Chapter 3). We observed here that Tamoxifen further reduced MCT1 gene expression in GPR81-silenced MCF-7 cells treated for 4 days **(Figure 4.6.A).** We next evaluated the effects of Tamoxifen and GPR81 silencing on cell proliferation and apoptosis in ER^+ MCF-7 breast cancer cells. GPR81 knockdown and Tamoxifen treatment had an additive effect on the reduction of cell proliferation markers; Ki67 and Cyclin B1 **(Figure 4.6.B),** as well as viable cell numbers of MCF-7 cells in MPM culture **(Figure 4.6. C).** Additionally, cell apoptosis was significantly increased only in MCF-7-siGPR81 cells independently of Tamoxifen treatment, showed by the increased expression of the apoptotic marker cleaved PARP. These data suggest GPR81 regulates cell proliferation and apoptosis.

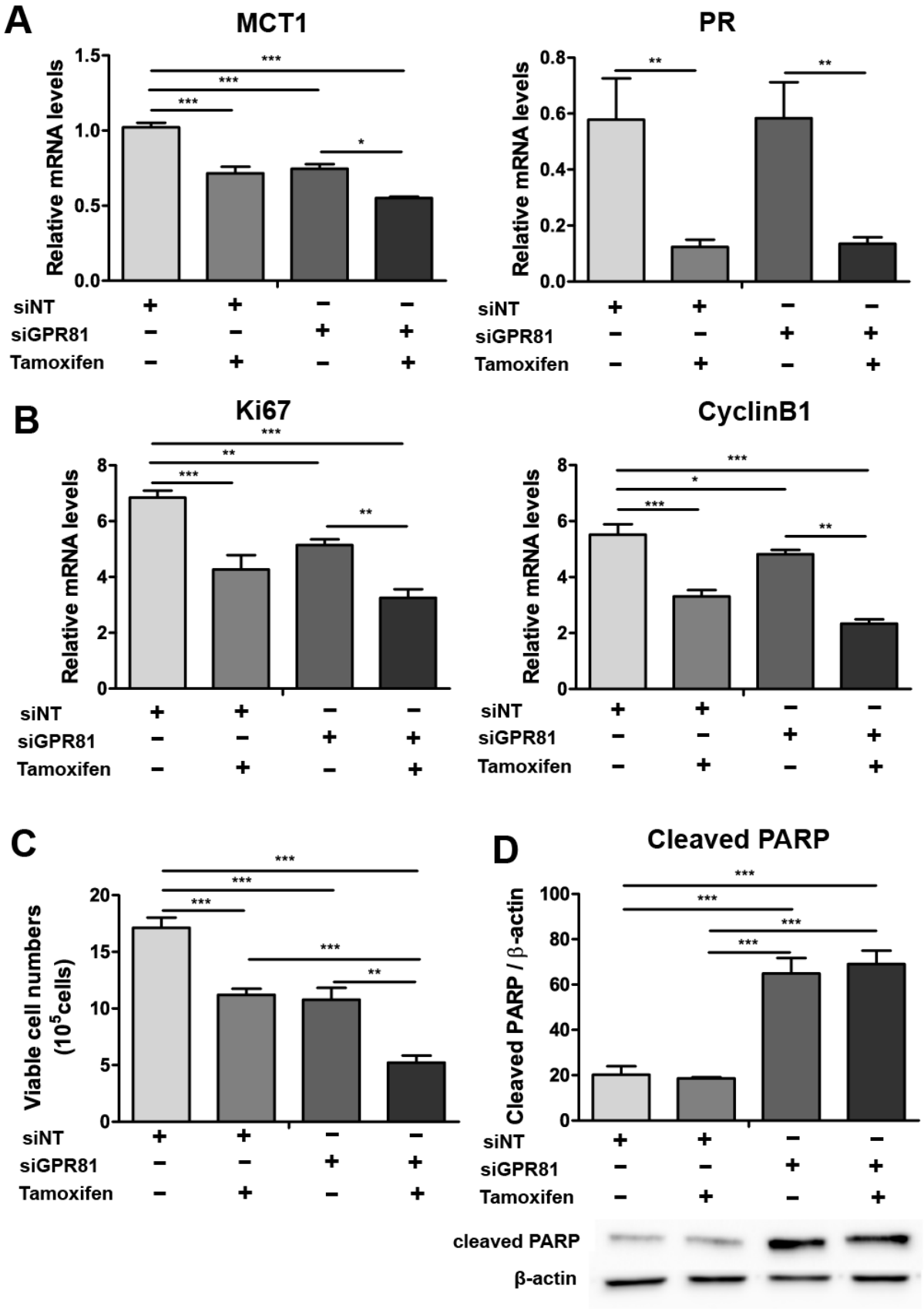

A
MCT1
Relative mRNA levels
siNT
siGPR81
Tamoxifen
PR
Relative mRNA levels
siNT
siGPR81
Tamoxifen
B
Ki67
Relative mRNA levels
siNT
siGPR81
Tamoxifen
CyclinB1
Relative mRNA levels
siNT
siGPR81
Tamoxifen
C
Viable cell numbers (10^5cells)
siNT
siGPR81
Tamoxifen
D
Cleaved PARP
Cleaved PARP / β-actin
siNT
siGPR81
Tamoxifen
cleaved PARP
β-actin

Figure 4.6. Additive effect of GPR81 knockdown and Tamoxifen treatment in reducing the cell proliferation and increasing cell apoptosis in epithelial MCF-7 breast cancer cells.

MCF-7-siNT (control) and MCF-7-siGPR81 cancer cells were treated with or without 1μM Tamoxifen for 96 hours in 3-dimensional Matrigel with physiological modified medium. **A.** Real-time qPCR analysis of lactate importer MCT1; and progesterone receptor PR. **B.** Relative mRNA expression of proliferation markers: Ki67 and Cyclin B1. **C.** Viable cell counts of epithelial MCF-7-siNT and MCF-7-siGPR81. **D.** Western blot analysis of cell lysates from MCF-7-siNT and MCF-7-siGPR81 used to detect protein expression levels of the apoptotic maker cleaved-PARP. β-actin was used as a loading control. Results are presented as mean ± S.E.M. of three independent experiments *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ (ordinary one-way ANOVA).

IV. Discussion

The metabolic changes that cancer cells undergo in order to support macromolecule biosynthesis, growth and survival, are collectively referred to as "metabolic reprogramming", now considered a hallmark of cancer cell biology[14,54]. Although there is increasing attention on cancer metabolic reprogramming, most studies have been performed in the setting of neoplastic transformation and less is known how metabolic reprogramming contributes to metastatic transformation. For example, it has been shown that the EMT-transcription factor SNAIL mediates the suppression of mitochondrial respiration and enhances glycolysis in breast cancer[58,73]. More recently, we demonstrated that EMT induced a metabolic shift to a less oxidative and more glycolytic metabolism in two epithelial cell lines, MCF-7 and BT-474, as evidenced by increased glucose uptake and lactate production along with increased expression of enzymes and transporters[47] (Chapter 3). These studies suggest that metastatic transformation or EMT facilitates the metabolic switch toward glycolysis.

Metabolic reprogramming induced by EMT was associated with a striking switch in the expression of the lactate transporters MCT1 and MCT4. Epithelial breast cancer cells express high levels of the lactate importer MCT1 and import lactate as an alternative energy source. In contrast, the mesenchymal cells showed a marked decreased expression of MCT1 and upregulation of the lactate exporter MCT4. This MCT4 upregulation was previously reported in triple negative breast cancer and high levels of this lactate exporter were associated with poor prognosis and survival[80,94]. As a result of the glycolytic shift induced by EMT, mesenchymal cells secrete higher amounts of lactate acid to the extracellular microenvironment which creates an acidic microenvironment that has been associated with tumor progression[19]. In addition, previous studies determined that extracellular lactate plays a critical role in cancer progression and metastasis by promoting cancer cell migration, immune escape and angiogenesis [31–33,35]. Most recently, lactate has been shown to act as a "signaling molecule", which, once imported by the cancer cell regulates intracellular signaling pathways like HIF1α-associated signaling[39], or binding directly to the metastatic activator NDRG3 (N-Myc Downstream Regulated Gene 3) [40]. In both cases a signaling cascade was triggered to promote tumor angiogenesis and tumor growth *in vivo*.

Lactate has been identified as the ligand for the endogenous cell-surface G-protein-coupled receptor 81 (GPR81). This lactate receptor was first discovered and mostly studied in adipocytes, in which lactate activates GPR81 to decrease the cAMP production to ultimately reduce lipolysis[42]. GPR81 is highly expressed in neurons[95], and when activated, it is able to modify electrical activity of primary neuronal cells[96]. Also, the activation of GPR81 by lactate has been identified as the initial step for an anti-inflammatory response in pancreas, liver and uterus[97–99].

More recently, GPR81 expression has been found to be upregulated in several types of cancer, including pancreatic, colon, liver, breast, lung and cervical cancers, and in several cases

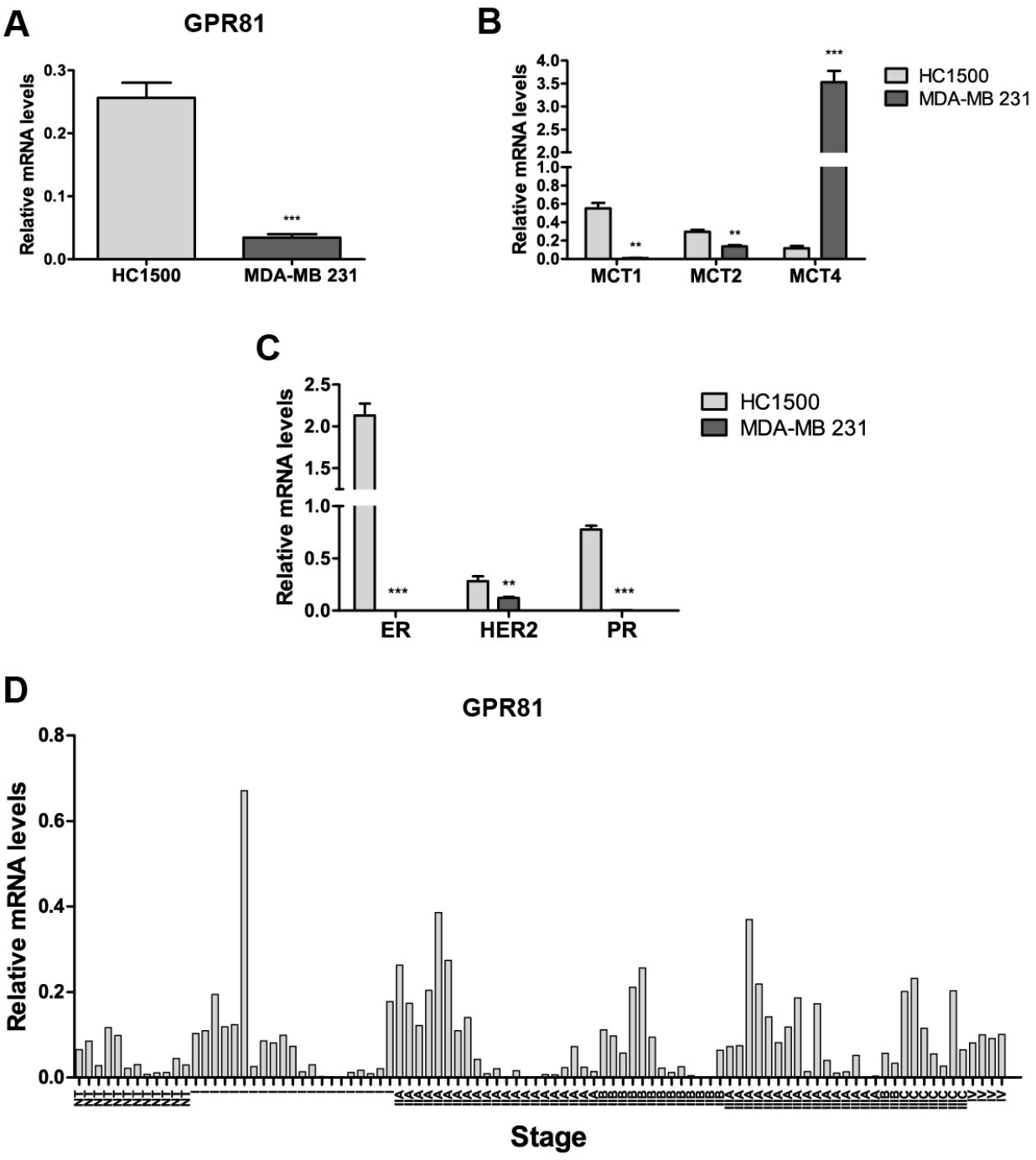
high GPR81 expression was associated with tumor growth, chemoresistance and metastasis[43–46]. Our study provides the first examination of GPR81 expression in the context of metastatic dissemination or EMT. We showed that hormone-positive epithelial breast cancer cells highly expressed GPR81, but after EMT induction and transformation into triple negative mesenchymal cancer cells, the expression of this lactate receptor was significantly reduced. Lee et al reported that GPR81 expression is significantly increased in breast cancer patients compared to normal mammary tissues[43]. We also observed a significant association between GPR81 overexpression with estrogen receptor (ERα)-positive and human epidermal growth human epidermal growth factor-2 receptor (HER2) positive human breast cancer tissues as opposed to triple negative breast cancer. This is consistent with previous observations that ERα-positive breast cancer tissues overexpressed GPR81[43]. In addition, GPR81 was expressed at higher levels in the first three stages of breast cancer (Stage I, II and III) as compared to Stage IV, and GPR81 expression correlated with better overall survival of breast cancer patients. These findings suggest that GPR81 may be an important regulator in hormone-positive breast cancer and could be used as a prognostic marker in the progression of breast cancer.

GPR81 was reported to regulate the expression of genes involved in lactate metabolism, including lactate transporters, MCT1 and MCT4, in pancreatic cancer cells[44]. In partial agreement, our study revealed that GPR81 specifically regulates the lactate importer MCT1, but not lactate exporter MCT4, in epithelial breast cancer cells. Oxidative breast tumor cells with high expression of the lactate importer, MCT1, have been reported to import and oxidize extracellular lactate, a mechanism that is essential for cell viability under glucose deprivation[28,93,100]. In fact, Park et al showed that epithelial breast cancer cells imported and utilized ^{14}C-lactate for mitochondrial respiration [43]. We previously showed that inhibition of the lactate importer, MCT1, reduced cell

proliferation in epithelial but not mesenchymal cancer cells when lactate was used as the primary metabolic substrate. Here we report that GPR81 silencing caused downregulation of MCT1 as well as reduced cell growth in epithelial breast cancer cells when grown in complete MPM medium. We previously reported that the two epithelial breast cancer cell lines contained significantly higher intracellular lactate and pyruvate than the two corresponding post-EMT mesenchymal cell lines. In the present study, we observed that GPR81-silenced cells had lower intracellular lactate and higher levels of lactate in MPM culture media compare to control cells, indicating reduction in lactate uptake. Furthermore, we found a protective effect of GPR81 against apoptosis, which was previously described in epithelial MCF-7 breast cancer cells, where GPR81 activation triggered the PI3K/Akt signaling pathway to inhibit apoptosis[43]. Together these results suggest a specific regulation of GPR81 on lactate importer MCT1, which affects cell proliferation, apoptosis and survival of hormone-positive epithelial breast cancer cells.

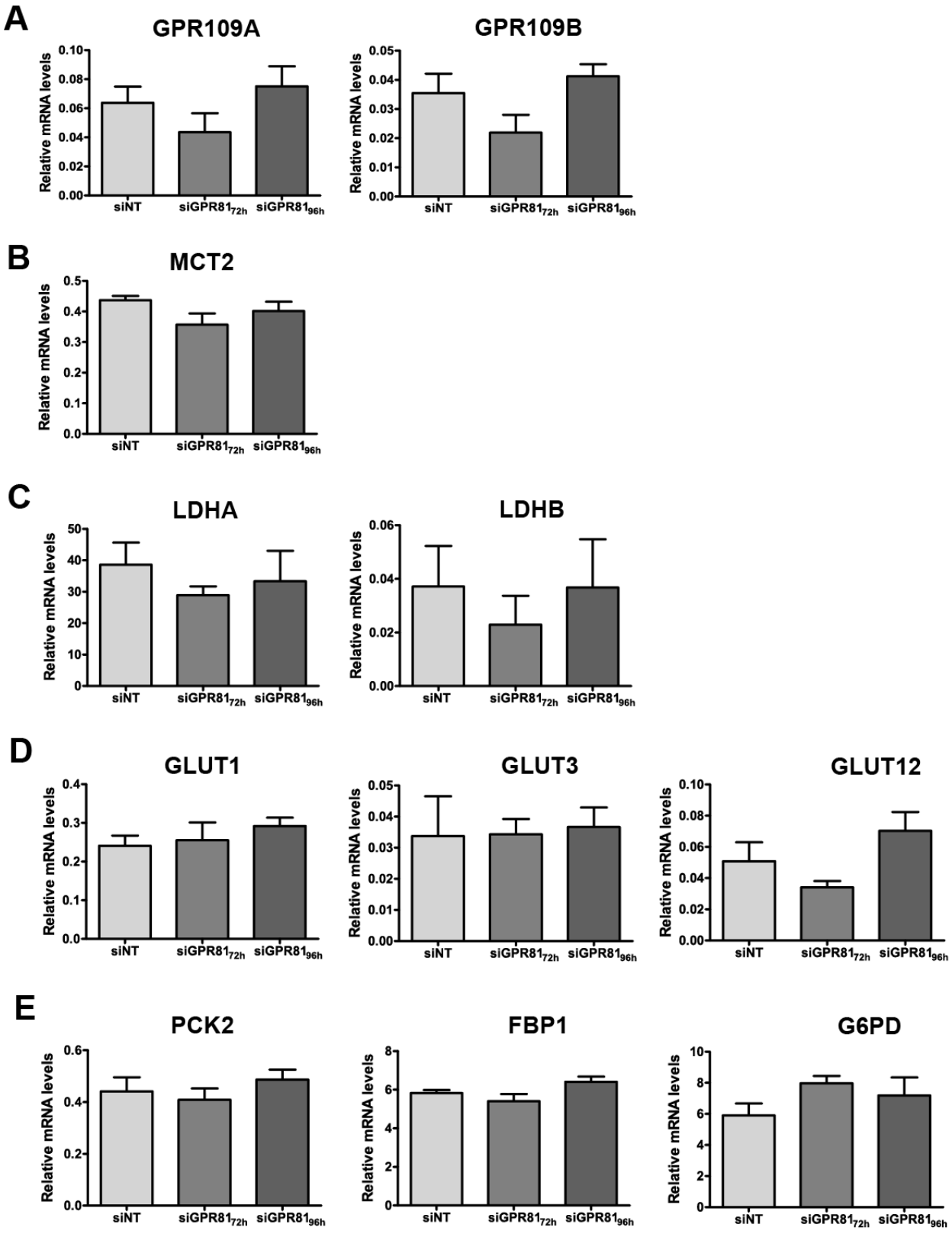
In adipocytes, GPR81 is coupled to Gi/q which inhibits adenylate cyclase activity and decreases the production of cAMP. However, a previous study did not find a significant change in cAMP levels when GPR81 was silenced in epithelial MCF-7 breast cancer cells[43], suggesting that GPR81 regulates MCT1 expression by another signaling pathway in these cells. It should also be noted that previous studies indicate that lactate activates GPR81 in the millimolar range (1 to 5 mM), and in human breast cancer tissues lactate was found to be at concentrations as high as 8 mM[28], suggesting that GPR81 and downstream signaling pathways could be constantly activated by lactate in the breast tumor microenvironment . Further studies are needed to examine which signaling pathways are linked to GPR81 in epithelial breast cancer, as these pathways may provide targets for future development of adjuvant therapies for Luminal A breast cancer.

V. Supplemental figures



Supplemental figure 4.1. GPR81 expression in breast cancer cell lines and tissue samples.

A. Real-time qPCR analysis of lactate receptor GPR81; lactate transporters: MCT1, MCT2 and MCT4 and hormone/growth factor receptors: ER, PR and HER2 in HC1500 and MDA-MB 231 breast cancer cell lines **B.** Relative mRNA expression of lactate receptor GPR81 in breast cancer tissue samples (n=74) and non-tumorigenic breast tissue (n=12). Results are presented as mean ± S.E.M. of three independent experiments *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ (ordinary one-way ANOVA).


A
GPR109A
GPR109B
Relative mRNA levels
siNT
siGPR81 72h
siGPR81 96h
B
MCT2
C
LDHA
LDHB
D
GLUT1
GLUT3
GLUT12
E
PCK2
FBP1
G6PD

Supplemental figure 4.2. GPR81 knockdown effect on relative mRNA expression of A. GPR81 homologous genes: GPR109A and GPR109B; **B.** lactate transporter MCT2; lactate dehydrogenases: LDHA and LDHB; glucose transporters: GLUT1, GLUT3 and GLUT12; and gluconeogenic enzymes: PCK2, FBP1 and G6PD in MCF-7-siNT (control) and MCF-7-siGPR81 cultured in 3-dimensional Matrigel with physiological modified medium at 72 and 96 hours after transfection. Results are presented as mean ± S.E.M. of three independent experiments *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ (ordinary one-way ANOVA).

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

I. Summary

Epithelial-mesenchymal transition (EMT) induces invasive properties in epithelial tumor cells and promotes metastasis. Previously, we induced EMT in two distinct epithelial breast cancer cell lines, MCF-7 and BT-474 cells that generated stable populations of mesenchymal cancer cells, termed MCF-7M and BT-474M cells, respectively[47,48]. A previous characterization of these cell lines using standard 2-dimensional culture on plastic dishes and a standard tissue culture medium, revealed that both epithelial MCF-7 and BT-474 cells are less glycolytic than the two mesenchymal MCF-7M and BT-474M cell lines[47]. In chapter 3 we examined the degree of oxidative vs glycolytic metabolism in these 4 cell lines, using a more physiological culture system that involves both a 3-dimensional Matrigel matrix and media containing the physiological levels of glucose, lactate, glutamine and pyruvate, termed MPM culture. Under these MPM culture conditions, both epithelial and mesenchymal cell lines maintained their phenotypic features and they showed marked morphological and behavioral differences. We further studied the metabolic shift induced by EMT using the Seahorse extracellular flux technology. We found that mesenchymal cancer cells had significant lower levels of oxygen consumption rate (OCR) and increased levels of extracellular acidification (ECAR) when compared to the parental epithelial cell lines, confirming a glycolytic shift induced by EMT. We further examined the metabolic differences in lactate flux and utilization by these four cell lines. We found that EMT induced overexpression of the lactate dehydrogenases LDHA and LDHB. In addition, EMT-induced mesenchymal cells downregulated the expression of the lactate importer MCT1 and overexpressed the lactate exporter MCT4. We also found that intracellular lactate and pyruvate were significantly lower in EMT-induced mesenchymal cancer cells. Together, these changes in lactate metabolism

induced by EMT support the glycolytic metabolism of mesenchymal cancer cells. In contrast, we found that oxidative epithelial cancer cells that highly expressed MCT1 are able to import lactate and use it as a nutrient source to support cell growth. Furthermore, we discovered that the transcription factor GATA3 and ERα signaling regulate the expression of the lactate importer MCT1, and the treatment with both Tamoxifen and MCT1 inhibitor has an additive effect on reducing cell growth of epithelial cancer cells.

In Chapter 4, we further continued our studies on lactate metabolism by examining the expression and role of the endogenous lactate receptor GPR81 in the context of EMT. We found that GPR81 is highly expressed in epithelial cancer cell lines but not in the derived EMT-induced mesenchymal cancer cell lines. Also, GPR81 overexpression was significantly associated with hormone-positive breast cancers and better overall survival of breast cancer patients. These findings suggest that GPR81 may be an important regulator in hormone-positive breast cancer and could be used as a prognostic marker in the progression of breast cancer. We observed that silencing of GPR81 downregulates the expression of the lactate importer MCT1, but not lactate exporter MCT4. In addition, GPR81 silencing caused decreased intracellular lactate, reduced cell proliferation and increased apoptosis in the epithelial cancer cells. Also, GPR81 expression was crucial for cell growth and survival of the epithelial cancer cells, when lactate was the only nutrient source available in the culture media. Together these findings suggest that GPR81 regulates lactate import through MCT1 expression by a mechanism yet to be elucidated. Furthermore, we found that Tamoxifen treatment and GPR81 silencing have an additive effect on reducing MCT1 expression and cell proliferation in epithelial breast cancer cells.

II. Future Directions

i. Lactate metabolism

We found that epithelial breast cancer cells exhibited an oxidative metabolism while the EMT-induced mesenchymal cancer cells have an enhanced glycolytic metabolism. To gain better insight into how both epithelial and mesenchymal cells metabolize glucose, we propose to perform glucose flux studies using ^{13}C-glucose and NMR. We will be able to determine the rates of glucose uptake, track the amount of glycolytic-derived lactate and other metabolites. We hypothesize that the rates of glucose uptake and glycolysis will be increased in the EMT-induced mesenchymal cancer cells.

We also found that epithelial breast cancer cells express high levels of the lactate importer MCT1 and the blockage of this lactate importer with MCT1 inhibitor reduced significantly their cell growth when lactate was only nutrient available. To confirm the ability of epithelial cancer cells to import lactate across the plasma membrane and use it to fuel their oxidative phosphorylation, we propose to perform lactate flux studies using ^{13}C-lactate and NMR. We propose to examine lactate uptake rates and lactate-derived metabolites by culturing the cells in glucose-deprived medium with ^{13}C-lactate and analysis of intracellular ^{13}C-lactate and ^{13}C-pyruvate by NMR. Also, we could use this approach to study how the blocking the lactate import, using treatments with MCT1 inhibitor or Tamoxifen or silencing of GATA3, affect the intracellular ^{13}C-lactate in epithelial breast cancer cells.

ii. Lactate transporters

We found high levels of lactate importer MCT1 in epithelial cancer cells, whereas EMT-induced mesenchymal cancer cells expressed high levels of lactate exporter MCT4, in either case

itis necessary to know whether MCT1 and MCT4 are functional transporters properly localized at the plasma membrane. We propose to examine the localization of these lactate transporters in epithelial and mesenchymal cancer cells using confocal microscopy and specific antibodies for MCT1 and MCT4.

Also, we found that the transcription factor GATA3 regulates the expression of the lactate importer MCT1 but not lactate exporter MCT4. We propose to confirm this interaction and regulation using a luciferase gene reporter assay with a vector containing the sequence for GATA3 binding site of the MCT1 promoter (with or without mutation) followed by the luciferase reporter gene. This approach will allow us to verify the transcriptional regulation of GATA3 on the gene expression of the lactate importer MCT1.

EMT induced the overexpression of the lactate exporter MCT4 in mesenchymal cancer cells. We propose to examine the effects of blocking MCT4 in EMT-induced mesenchymal cells using specific MCT4 inhibitors. In addition, we propose to study MCT4 regulation in EMT-induced mesenchymal cells using a signaling pathway panel of inhibitors containing 12 to 16 inhibitors to elucidate a specific signaling cascade.

Finally, it will be important to examine the effects of both knockdown and overexpression of MCT1 and MCT4, along with inhibitors of MCT1 and MCT4, on cell proliferation, growth and possibly metabolic adaptations in the context of orthologous transplantation of cells into the mammary fat pads of immunodeficient mice.

iii. Lactate receptor GPR81

We propose to study the localization of the lactate receptor GPR81 in epithelial and mesenchymal breast cancer cells by transfecting them with the plasmid pCMV6-AC-GFP (Origene), which contains the GPR81 ORF sequence tagged with GFP at the C-terminal region

(GPR81-GFP). Our preliminary studies using these plasmids indicate that GPR81 localizes at the plasma membrane in epithelial breast cancer cells but GPR81 is restricted to the nucleus in EMT-induced breast cancer cells. Further studies are needed to confirm the localization of GPR81 in these specific compartments in the cell using control proteins at the plasma membrane or nucleus.

We found a significant association between hormone-positive breast cancer tissues and cells with overexpression of GPR81. Further studies would examine whether epithelial breast cancer-related transcription factors (e.g., ERα, GATA3, FOXA1) stimulate GPR81 gene transcription.

Previous studies on normal cells (e.g., adipocytes) revealed that the lactate receptor GPR81 blocks adenyl cyclase to lower the levels of cAMP and increase intracellular Ca^{+2} levels. Although, previous studies showed no change in cAMP levels when GPR81 was silenced epithelial cancer cells[43], we propose to attempt to reproduce this finding in epithelial cancer cells culture in MPM.

If we also observe no changes in cAMP levels in response to GPR81 knockdown or overexpression, we propose to use pharmacological and siRNA screens to identify the signaling pathway(s) that is/are coupled to GPR81.

APPENDIX

Appendix: Primer sets for real-time qPCR

GENE SYMBOL	FORWARD PRIMER	REVERSE PRIMER
TBP	TTGCTGCGGTAATCATGAGG	TTTTCTTGCTGCCAGTCTGG
ZEB1	TGCACAAGAAGAGCCACAAG	TGCGCAAGACAAGTTCAAGG
ZEB2	ATGCTTTTGCCCAACTGCTG	ACAGAGAGGGCAGGAAAAGTTC
CDH1	TTGAACGAATGGGGCAATCG	ACCAGCAACGTGATTTCTGC
GATA3	ATGAAGCCTAAACGCGATGG	TGAGCTCCTTTGCAAAGTGG
VIM	TCTCAGCATCACGATGACCTTG	TTGCGCTCCTGAAAAACTGC
CD24	AACTAATGCCACCACCAAGG	ATGCAGAAGAGAGAGTGAGACC
CD44	AAGGTGGAGCAAACACAACC	TCGACTGTTGACTGCAATGC
GPR81	TCATTGTGGCCTTTGTGCTG	AATCAGCCACGGCCAAATTG
GPR109A	AAGATCTCCAATCGGACAGCAG	AATTTGCACCGCCATTCTGG
GPR109B	TGGTGCTCTTCATGTTTGCC	TGATGGCTGCTGTCCAATTG
ERα	TTGAAACACAAGCGCCAGAG	TTTGATCATGAGCGGGCTTG
PR	TGCAGACTTTACAGGCAAGC	AGGGCAAAGAGGACAAATGC
HER2	TTTTGGGGCCAAACCTTACG	ACATCAATGGTGCAGATGGG
Cyclin B1	AAGGCGAAGATCAACATGGC	TTTGGCCTGCAGTTGTTCAC
KI67	TGCAGCAAAACAGCCATCTG	TTCCTGGGCGTTTTTGCTAC
PCNA	AACCTGCAGAGCATGGACTC	TCATTGCCGGCGCATTTTAG
STK15	AATTCTTCCCAGCGCATTCC	TGGTTGCCTGCAATTGCTTC
MCT1	TGGTGACCATTGTGGAATGC	AATTAGGACGACGCCACATG
MCT2	AGCAGAAATGCCACCAATGC	AAGCTGCTCCAACCACAATC
MCT4	TTCGGCTGTTTCGTCATCAC	TCCCAAACTCCTGTATGAGCTC
LDHA	CATGGCAGCCTTTTCCTTAG	ATGACCAGCTTGGAGTTTGC
LDHB	AACATGGCGACTCAAGTGTG	AGGCACTTTCAACCACCATC
PCK2	TGCGTATTATGACCCGACTG	TCAGGGTTTTCTCTGGGTTG
FBP1	AAGTCATCCTTTGCCACGTG	TTTACCCCTTTTCTCCGGTTCC
G6PD	TGCCCGAAAACACCTTCATC	TCACTCTGTTTGCGGATGTC
GLUT1	ATGCCGATTTGGTTCCTGTG	TTTTCTGAGTGCCTGCTGTG
GLUT3	TTGACGGACAAGGGAAATGC	TTGAATTGCGCCTGCCAAAG
GLUT12	AAAGCAATGAGGCAGCTAGC	ATGTTTTGCTGCCGACATGG

REFERENCES

1. Hassiotou, F. & Geddes, D. Anatomy of the human mammary gland: Current status of knowledge. *Clin. Anat.* **26,** 29–48 (2013).
2. Zucca-Matthes, G., Urban, C. & Vallejo, A. Anatomy of the nipple and breast ducts. *Gland Surg.* **5,** 32–6 (2016).
3. Javed, A. & Lteif, A. Development of the human breast. *Semin. Plast. Surg.* **27,** 5–12 (2013).
4. Gusterson, B. A. & Stein, T. Human breast development. *Semin. Cell Dev. Biol.* **23,** 567–573 (2012).
5. Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics. *CA Cancer J Clin* **66,** 7–30 (2016).
6. Makki, J. Diversity of Breast Carcinoma : Histological Subtypes and Clinical Relevance. *Clin. Med. Insights Pathol.* 23–31 (2015). doi:10.4137/CPath.S31563.TYPE
7. Eliyatkin, N., Yalcin, E., Zengel, B., Aktaş, S. & Vardar, E. Molecular Classification of Breast Carcinoma: From Traditional, Old-Fashioned Way to A New Age, and A New Way. *J. Breast Heal.* **11,** 59–66 (2015).
8. Nieto, M. A., Huang, R. Y. J., Jackson, R. A. & Thiery, J. P. EMT: 2016. *Cell* **166,** 21–45 (2016).
9. Kalluri, R. & Weinberg, R. a. Review series The basics of epithelial-mesenchymal transition. *J. Clin. Invest.* **119,** 1420–1428 (2009).
10. Tania, M., Khan, M. A. & Fu, J. Epithelial to mesenchymal transition inducing transcription factors and metastatic cancer. *Tumour Biol.* **35,** 7335–42 (2014).
11. Lamouille, S., Xu, J. & Derynck, R. Molecular mechanisms of epithelial–mesenchymal transition. *Nat. Rev. Mol. Cell Biol.* **15,** 178–196 (2014).
12. Felipe Lima, J., Nofech-Mozes, S., Bayani, J. & Bartlett, J. M. S. EMT in Breast Carcinoma-A Review. *J. Clin. Med.* **5,** 1–14 (2016).
13. Gonzalez, D. M. & Medici, D. Signaling mechanisms of the epithelial-mesenchymal transition. **7,** 1–17 (2014).
14. Pavlova, N. N. & Thompson, C. B. The Emerging Hallmarks of Cancer Metabolism. *Cell Metab.* **23,** 27–47 (2016).
15. Agathocleous, M. & Harris, W. a. Metabolism in physiological cell proliferation and differentiation. *Trends Cell Biol.* **23,** 484–92 (2013).
16. Ward, P. S. & Thompson, C. B. Metabolic Reprogramming: A Cancer Hallmark Even Warburg

Did Not Anticipate. *Cancer Cell* **21,** 297–308 (2012).

17. Hernández-Reséndiz, I. *et al.* Dual regulation of energy metabolism by p53 in human cervix and breast cancer cells. *Biochim. Biophys. Acta - Mol. Cell Res.* **1853,** 3266–3278 (2015).
18. Vander Heiden, M. G. *et al.* Metabolic pathway alterations that support cell proliferation. *Cold Spring Harb. Symp. Quant. Biol.* **76,** 325–34 (2011).
19. Kato, Y. *et al.* Acidic extracellular microenvironment and cancer. *Cancer Cell Int.* **13,** 89 (2013).
20. Birsoy, K. *et al.* Metabolic determinants of cancer cell sensitivity to glucose limitation and biguanides. *Nature* **508,** 108–112 (2014).
21. LeBleu, V. S. *et al.* PGC-1α mediates mitochondrial biogenesis and oxidative phosphorylation in cancer cells to promote metastasis. *Nat. Cell Biol.* **16,** 992–1003 (2014).
22. Feron, O. Pyruvate into lactate and back: from the Warburg effect to symbiotic energy fuel exchange in cancer cells. *Radiother. Oncol.* **92,** 329–33 (2009).
23. Fu, Y. *et al.* The reverse Warburg effect is likely to be an Achilles' heel of cancer that can be exploited for cancer therapy. *Oncotarget* **5,** 57813–57825 (2015).
24. Migneco, G. *et al.* Glycolytic cancer associated fibroblasts promote breast cancer tumor growth, without a measurable increase in angiogenesis: evidence for stromal-epithelial metabolic coupling. *Cell Cycle* **9,** 2412–22 (2010).
25. Doherty, J. R. & Cleveland, J. L. Targeting lactate metabolism for cancer therapeutics. *J. Clin. Invest.* **123,** 3685–92 (2013).
26. Halestrap, A. P. The monocarboxylate transporter family-Structure and functional characterization. *IUBMB Life* **64,** 1–9 (2012).
27. Sonveaux, P. *et al.* Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. *J. Clin. Invest.* **118,** 3930–3942 (2008).
28. Kennedy, K. M. *et al.* Catabolism of exogenous lactate reveals it as a legitimate metabolic substrate in breast cancer. *PLoS One* **8,** e75154 (2013).
29. Sotgia, F. *et al.* Mitochondria 'fuel' breast cancer metabolism: fifteen markers of mitochondrial biogenesis label epithelial cancer cells, but are excluded from adjacent stromal cells. *Cell Cycle* **11,** 4390–401 (2012).
30. Martinez-Outschoorn, U. E. *et al.* Ketones and lactate increase cancer cell 'stemness,' driving recurrence, metastasis and poor clinical outcome in breast cancer: achieving personalized medicine via Metabolo-Genomics. *Cell Cycle* **10,** 1271–86 (2011).
31. Goetze, K., Walenta, S., Ksiazkiewicz, M., Kunz-Schughart, L. a & Mueller-Klieser, W. Lactate enhances motility of tumor cells and inhibits monocyte migration and cytokine release. *Int. J. Oncol.* **39,** 453–63 (2011).

32. Baumann, F. *et al.* Lactate promotes glioma migration by TGF-beta2-dependent regulation of matrix metalloproteinase-2. *Neuro. Oncol.* **11,** 368–80 (2009).
33. Colegio, O. R. *et al.* Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature* **513,** 559–63 (2014).
34. Ziebart, T. *et al.* Metabolic and proteomic differentials in head and neck squamous cell carcinomas and normal gingival tissue. *J. Cancer Res. Clin. Oncol.* **137,** 193–9 (2011).
35. Végran, F., Boidot, R., Michiels, C., Sonveaux, P. & Feron, O. Lactate influx through the endothelial cell monocarboxylate transporter MCT1 supports an NF-kB/IL-8 pathway that drives tumor angiogenesis. *Cancer Res.* **71,** 2550–2560 (2011).
36. Luc, R., Tortorella, S. M., Ververis, K. & Karagiannis, T. C. Lactate as an insidious metabolite due to the Warburg effect. *Mol. Biol. Rep.* **42,** 835–40 (2015).
37. Hirschhaeuser, F., Sattler, U. G. a & Mueller-Klieser, W. Lactate: a metabolic key player in cancer. *Cancer Res.* **71,** 6921–5 (2011).
38. Doherty, J. R. *et al.* Blocking lactate export by inhibiting the Myc target MCT1 Disables glycolysis and glutathione synthesis. *Cancer Res.* **74,** 908–20 (2014).
39. de Saedeleer, C. J. *et al.* Lactate Activates HIF-1 in Oxidative but Not in Warburg-Phenotype Human Tumor Cells. *PLoS One* **7,** (2012).
40. Lee, D. C. *et al.* A lactate-induced response to hypoxia. *Cell* **161,** 595–609 (2015).
41. Liu, C. *et al.* Lactate inhibits lipolysis in fat cells through activation of an orphan G-protein-coupled receptor, GPR81. *J. Biol. Chem.* **284,** 2811–22 (2009).
42. Ahmed, K. *et al.* An autocrine lactate loop mediates insulin-dependent inhibition of lipolysis through GPR81. *Cell Metab.* **11,** 311–9 (2010).
43. Lee, Y. J. *et al.* G-protein-coupled receptor 81 promotes a malignant phenotype in breast cancer through angiogenic factor secretion. *Oncotarget* **7,** 70898–70911 (2016).
44. Roland, C. L. *et al.* Cell Surface Lactate Receptor GPR81 Is Crucial for Cancer Cell Survival. *Cancer Res.* **74,** 5301–10 (2014).
45. Wagner, W., Kania, K. D. & Ciszewski, W. M. Stimulation of lactate receptor (HCAR1) affects cellular DNA repair capacity. *DNA Repair (Amst).* **52,** 49–58 (2017).
46. Wagner, W., Ciszewski, W. M. & Kania, K. D. L- and D-lactate enhance DNA repair and modulate the resistance of cervical carcinoma cells to anticancer drugs via histone deacetylase inhibition and hydroxycarboxylic acid receptor 1 activation. *Cell Commun. Signal.* **13,** 36 (2015).
47. Kondaveeti, Y., Guttilla Reed, I. K. & White, B. a. Epithelial-mesenchymal transition induces similar metabolic alterations in two independent breast cancer cell lines. *Cancer Lett.* **364,** 44–58 (2015).

48. Guttilla, I. K. *et al.* Prolonged mammosphere culture of MCF-7 cells induces an EMT and repression of the estrogen receptor by microRNAs. *Breast Cancer Res. Treat.* **132,** 75–85 (2012).
49. Lee, G. Y., Kenny, P. A., Lee, E. H. & Bissell, M. J. Three-dimensional culture models of normal and malignant breast epithelial cells. *Nat. Methods* **4,** 359–365 (2007).
50. Bi, H. *et al.* Optimization of harvesting, extraction, and analytical protocols for UPLC-ESI-MS-based metabolomic analysis of adherent mammalian cancer cells. *Anal. Bioanal. Chem.* **405,** 5279–5289 (2013).
51. Torre, L. A. *et al.* Global Cancer Statistics, 2012. *CA a cancer J. Clin.* **65,** 87–108 (2015).
52. Chaffer, C. L. & Weinberg, R. a. A perspective on cancer cell metastasis. *Science* **331,** 1559–1564 (2011).
53. Cantor, J. R. & Sabatini, D. M. Cancer cell metabolism: one hallmark, many faces. *Cancer Discov.* **2,** 881–98 (2012).
54. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: The next generation. *Cell* **144,** 646–674 (2011).
55. Chang, C.-C. *et al.* Upregulation of lactate dehydrogenase a by 14-3-3ζ leads to increased glycolysis critical for breast cancer initiation and progression. *Oncotarget* **7,** 35270–83 (2016).
56. Brisson, L. *et al.* Lactate Dehydrogenase B Controls Lysosome Activity and Autophagy in Cancer. *Cancer Cell* **30,** 418–431 (2016).
57. Penkert, J. & Ripperger, T. On metabolic reprogramming and tumor biology: A comprehensive survey of metabolism in breast cancer. *Oncotarget* **7,** (2016).
58. Dong, C. *et al.* Loss of FBP1 by Snail-mediated repression provides metabolic advantages in basal-like breast cancer. *Cancer Cell* **23,** 316–31 (2013).
59. Bhowmik, S., Ramirez-Peña, E. & Arnold, J. EMT-induced metabolite signature identifies poor clinical outcome. *Oncotarget* **6,** (2015).
60. Krebsbach, P. H. & Villa-Diaz, L. G. The role of integrin α6 (CD49f) in stem cells: more than a conserved biomarker. *Stem Cells Dev.* **0,** scd.2016.0319 (2017).
61. Soady, K. J. *et al.* Mouse mammary stem cells express prognostic markers for triple-negative breast cancer. *Breast Cancer Res.* **17,** 31 (2015).
62. Tsutsui, S. *et al.* A loss of c-kit expression is associated with an advanced stage and poor prognosis in breast cancer. *Br. J. Cancer* **94,** 1874–1878 (2006).
63. Chou, J., Provot, S. & Werb, Z. GATA3 in development and cancer differentiation: Cells GATA have it! *J. Cell. Physiol.* **222,** 42–49 (2010).
64. Fang, S. H., Chen, Y. & Weigel, R. J. GATA-3 as a marker of hormone response in breast cancer. *J. Surg. Res.* **157,** 290–5 (2009).

65. Lesurf, R. *et al.* ORegAnno 3.0: A community-driven resource for curated regulatory annotation. *Nucleic Acids Res.* **44,** D126–D132 (2016).
66. Eeckhoute, J. *et al.* Positive cross-regulatory loop ties GATA-3 to estrogen receptor α expression in breast cancer. *Cancer Res.* **67,** 6477–6483 (2007).
67. Hurtado, A., Holmes, K. A., Ross-Innes, C. S., Schmidt, D. & Carroll, J. S. FOXA1 is a key determinant of estrogen receptor function and endocrine response. *Nat. Genet.* **43,** 27–33 (2011).
68. Anastasiou, D. Tumour microenvironment factors shaping the cancer metabolism landscape. *Br. J. Cancer* **116,** 277–286 (2016).
69. Nagelkerke, A., Bussink, J., Rowan, A. E. & Span, P. N. The mechanical microenvironment in cancer: How physics affects tumours. *Semin. Cancer Biol.* **35,** 62–70 (2015).
70. Diers, A. R., Broniowska, K. a, Chang, C.-F. & Hogg, N. Pyruvate fuels mitochondrial respiration and proliferation of breast cancer cells: effect of monocarboxylate transporter inhibition. *Biochem. J.* **444,** 561–71 (2012).
71. Keenan, M. M. & Chi, J.-T. Alternative fuels for cancer cells. *Cancer J.* **21,** 49–55 (2015).
72. Elia, I. *et al.* Proline metabolism supports metastasis formation and could be inhibited to selectively target metastasizing cancer cells. *Nat. Commun.* **8,** 15267 (2017).
73. Lee, S. Y. *et al.* Wnt/snail signaling regulates cytochrome c oxidase and glucose metabolism. *Cancer Res.* **72,** 3607–3617 (2012).
74. Zhao, D. *et al.* Lysine-5 acetylation negatively regulates lactate dehydrogenase A and is decreased in pancreatic cancer. *Cancer Cell* **23,** 464–76 (2013).
75. Koukourakis, M. I. *et al.* Lactate dehydrogenase 5 expression in squamous cell head and neck cancer relates to prognosis following radical or postoperative radiotherapy. *Oncology* **77,** 285–292 (2009).
76. Sun, X. *et al.* Expression of SIP1 is strongly correlated with LDHA and shows a significantly poor outcome in gastric cancer. *Tumor Biol.* **36,** 7521–7530 (2015).
77. Dennison, J. B. *et al.* Lactate dehydrogenase B: a metabolic marker of response to neoadjuvant chemotherapy in breast cancer. *Clin. Cancer Res.* **19,** 3703–13 (2013).
78. McCleland, M. L. *et al.* An integrated genomic screen identifies LDHB as an essential gene for triple-negative breast cancer. *Cancer Res.* **72,** 5812–5823 (2012).
79. Pérez-Escuredo, J. *et al.* Monocarboxylate transporters in the brain and in cancer. *Biochim. Biophys. Acta - Mol. Cell Res.* **1863,** 2481–2497 (2016).
80. Doyen, J. *et al.* Expression of the hypoxia-inducible monocarboxylate transporter MCT4 is increased in triple negative breast cancer and correlates independently with clinical outcome. *Biochem. Biophys. Res. Commun.* **451,** 54–61 (2014).

81. Pinheiro, C. *et al.* Monocarboxylate transporter 1 is up-regulated in basal-like breast carcinoma. *Histopathology* **56,** 860–867 (2010).
82. Johnson, J. M. *et al.* MCT1 in Invasive Ductal Carcinoma: Monocarboxylate Metabolism and Aggressive Breast Cancer. *Front. Cell Dev. Biol.* **5,** 1–8 (2017).
83. Hong, C. S. *et al.* MCT1 Modulates Cancer Cell Pyruvate Export and Growth of Tumors that Co-express MCT1 and MCT4. *Cell Rep.* **14,** 1590–1601 (2016).
84. Pisarsky, L. *et al.* Targeting Metabolic Symbiosis to Overcome Resistance to Anti-angiogenic Therapy. *Cell Rep.* **15,** 1161–74 (2016).
85. San-Millán, I. & Brooks, G. A. Reexamining cancer metabolism: Lactate production for carcinogenesis could be the purpose and explanation of the Warburg Effect. *Carcinogenesis* **38,** 119–133 (2017).
86. Walenta, S. *et al.* High Lactate Levels Predict Likelihood of Metastases , Tumor Recurrence , and Restricted Patient Survival in Human Cervical Cancers 1. 916–921 (2000).
87. Doherty, J. R. *et al.* Blocking lactate export by inhibiting the Myc target MCT1 Disables glycolysis and glutathione synthesis. *Cancer Res.* **74,** 908–20 (2014).
88. Park, J. *et al.* Cancer cells induce metastasis-supporting neutrophil extracellular DNA traps. *Sci. Transl. Med.* **8,** 361ra138-361ra138 (2016).
89. Sarah E. R. Halford, Paul Jones, Steve Wedge, Sandra Hirschberg, Sidath Katugampola, Gareth Veal, Geoffrey Payne, Chris Bacon, Sarah Potter, Melanie Griffin, Maxime Chenard-Poirier, George Petrides, Graham Holder, Hector C Keun, Udai Banerji, and E. R. P. A first-in-human first-in-class (FIC) trial of the monocarboxylate transporter 1 (MCT1) inhibitor AZD3965 in patients with advanced solid tumours.No Title. *J. Clin. Oncol.* **35,** 2516–2516 (2017).
90. Morais-Santos, F. *et al.* Targeting lactate transport suppresses in vivo breast tumour growth. *Oncotarget* **6,** 19177–19189 (2015).
91. Stäubert, C., Broom, O. J. & Nordström, A. Hydroxycarboxylic acid receptors are essential for breast cancer cells to control their lipid / fatty acid metabolism. **6,** (2015).
92. Lánczky A, Nagy Á, Bottai G, Munkácsy G, Szabó A, Santarpia L, G. B. miRpower: a web-tool to validate survival-associated miRNAs utilizing expression data from 2178 breast cancer patients. *Breast Cancer Res. Treat.* **160,** 439–446 (2016).
93. Park, S. *et al.* ERRα-Regulated Lactate Metabolism Contributes to Resistance to Targeted Therapies in Breast Cancer. *Cell Rep.* **15,** 323–35 (2016).
94. Baenke, F. *et al.* Functional screening identifies MCT4 as a key regulator of breast cancer cell metabolism and survival. *J. Pathol.* **237,** 152–165 (2015).
95. Lauritzen, K. H. *et al.* Lactate receptor sites link neurotransmission, neurovascular coupling, and

brain energy metabolism. *Cereb. Cortex* **24,** 2784–2795 (2014).

96. Bozzo, L., Puyal, J. & Chatton, J.-Y. Lactate Modulates the Activity of Primary Cortical Neurons through a Receptor-Mediated Pathway. *PLoS One* **8,** e71721 (2013).
97. Lerch, M. M., Conwell, D. L. & Mayerle, J. The anti-inflammasome effect of lactate and the lactate GPR81-receptor in pancreatic and liver inflammation. *Gastroenterology* **146,** 1602–5 (2014).
98. Madaan, A. *et al.* Lactate produced during labor modulates uterine inflammation via GPR81 (HCA1). *Am. J. Obstet. Gynecol.* **216,** 60.e1-60.e17 (2017).
99. Hoque, R., Farooq, A., Ghani, A., Gorelick, F. & Mehal, W. Z. Lactate reduces liver and pancreatic injury in toll-like receptor- and inflammasome-mediated inflammation via gpr81-mediated suppression of innate immunity. *Gastroenterology* **146,** 1763–1774 (2014).
100. Boidot, R. *et al.* Regulation of monocarboxylate transporter MCT1 expression by p53 mediates inward and outward lactate fluxes in tumors. *Cancer Res.* **72,** 939–948 (2012).